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ORIGINAL PAPER

Diversity of Culturable Bacteria Isolated from Highland Barley Cultivation Soil in Qamdo, Tibet Autonomous Region

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Abstract

The soil bacterial communities have been widely investigated. However, there has been little study of the bacteria in Qinghai-Tibet Plateau, especially about the culturable bacteria in highland barley cultivation soil. Here, a total of 830 individual strains were obtained at 4°C and 25°C from a highland barley cultivation soil in Qamdo, Tibet Autonomous Region, using fifteen kinds of media. Seventy-seven species were obtained, which belonged to 42 genera and four phyla; the predominant phylum was Actinobacteria (68.82%), followed by Proteobacteria (15.59%), Firmicutes (14.29%), and Bacteroidetes (1.30%). The predominant genus was *Streptomyces* (22.08%, 17 species), followed by *Bacillus* (6.49%, five species), *Micromonospora* (5.19%, four species), *Microbacterium* (5.19%, four species), and *Kribbella* (3.90%, three species). The most diverse isolates belonged to a high G+C Gram-positive group; in particular, the *Streptomyces* genus is a dominant genus in the high G+C Gram-positive group. There were 62 species and 33 genera bacteria isolated at 25°C (80.52%), 23 species, and 18 genera bacteria isolated at 4°C (29.87%). Meanwhile, only eight species and six genera bacteria could be isolated at 25°C and 4°C. Of the 77 species, six isolates related to six genera might be novel taxa. The results showed abundant bacterial species diversity in the soil sample from the Qamdo, Tibet Autonomous Region.

K e y w o r d s: Qinghai-Tibet Plateau, *Streptomyces*, 16S rRNA, novel taxa, high-altitude area

Introduction

Bacteria constitute a major proportion of biodiversity in soil ecosystems; they are the main driving force for the conversion and circulation of carbon, nitrogen, and phosphorus, and also the prominent participants in biochemical processes of soil organic matter decomposition and humus formation (Fulthorpe et al. 2008; Řeháková et al. 2015; Malard et al. 2019). Bacterial assemblages are essential components of soils in arid ecosystems, especially in remote high-elevation mountains (Margesin et al. 2009; Yuan et al. 2014). While global surveys of microbial diversity and functional activity have already been conducted (Bodelier 2011; Delgado-Baquerizo et al. 2018), the number of Qinghai-Tibet Plateau samples is restricted, and, therefore bacterial data is still lacking in this area, especially in the most high-altitude area (Zhang et al. 2016).

Highland barley (*Hordeum vulgare* L.) is the fourth most consumed grain worldwide, only ranked after rice, wheat, and maize (Shen et al. 2016; Deng et al. 2020). Highland barley is a hulless barley cultivar and used as the main staple food for the Tibetan people widely grown in Qinghai-Tibet Plateau in China (He et al. 2019; Zhang et al. 2019). Extreme environments such as cold and hypoxia in Tibet have promoted the unique ecological environment and soil bacterial composition (Zhang et al. 2007; 2010a). However, the extreme environments also have led to the decline of soil bacterial activity and the impoverishment of soil for growing highland barley (Yu et al. 2009; Zhao et al. 2014). The research of soil bacteria in the highland barley planting field has important significance for highland barley yield increase, pest control, and soil quality improvement (Bailly and Weisskopf 2012). At present, there were few studies on bacteria in the soil of the highland

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barley-planting field (Liu et al. 2019). Significantly, the culturable bacteria isolated from highland barley cultivation soil have not been reported systematically.

The Qamdo region's temperature is between 20°C and 28°C from June to September, a significant growth period for highland barley. While the temperature is below 10°C from November to March, no crops were planted on the land during this period. So the culturable bacteria were isolated from a high-altitude highland barley cultivation soil collected in Qamdo using 15 media at 4°C and 25°C to simulate the temperature conditions over these two periods in this study. The composition of bacterial communities was characterized based on the 16S rRNA gene (Furlong et al. 2002; Li et al. 2019). Our aims were: (1) to reveal the diversity of culturable bacteria isolated from highland barley cultivation soil in the high-altitude area; and (2) to study the effect of different culture temperatures on the species of culturable bacteria in highland barley cultivation soil.

Experimental

Materials and Methods

Study site and samples collection. The sampling site was located in the Zhu Village, Banbar County, Qamdo, Tibet Autonomous Region (30°55'48.9'N, 94°58'13.4'E, Altitude: 4,011 m); the sampling site is the typical high-altitude patches farmland in Qamdo, which is about one-third of Qamdo's farmland. The sample site belongs to the plateau temperate subhumid climate type, the air temperature range is –40–29°C, the annual average air temperature is –1°C, and the yearly frozen period is from September to April. The soil type was sandy loam, and the pH value is 7.6. The previous crop was highland barley, and the yield is about 1,000–1,800 kg/hm^2 in this area. A highland barley cultivation soil sample was collected from a depth of 5–15 cm using the five-point method and kept in sterilized paper bags in April 2018. Once retrieved, the soil sample was immediately stored at 4°C, and bacteria were isolated in the laboratory in Lhasa in May and June 2018.

Isolation and maintenance of bacteria. The bacteria in highland barley cultivation soil sample were isolated using X1, R, L1, ISP2, GW1, DSM372, F1, F2, M1, M5, M6, M7, M8, HV, and GS media, as shown in Table I. Gram-negative bacteria and Actinobacteria were isolated by using the dilution plating technique as described by Kuklinsky-Sobral et al. (2004) and Zhang et al. (2016), respectively, with some modifications. 0.2 ml of 10^{-2}, 10^{-3}, and 10^{-4} soil suspensions were spread onto F1, F2, M1, M5, M6, M7, M8, HV, and GS media to isolate Actinobacteria. While, 0.2 ml of 10^{-4}, 10^{-5}, and 10^{-6} soil suspension was spread onto X1, R, L1, ISP2, GW1, and DSM372 media to isolate Gram-negative bacteria. Two sets of plates were incubated at 4°C and 25°C, respectively; the bacterial strains were obtained across 3–60 days. The pure culture isolates were preserved in glycerol suspensions (20%, v/v) at –80°C for further research.

PCR amplification and sequencing of the 16S rRNA gene. According to the manufacturer's protocol, the genomic DNA of bacteria was extracted using a bacterial genomic DNA FastPrep Extraction Kit (TIANGEN DP302). Polymerase chain reaction (PCR) amplification of the partial 16S rRNA gene was performed using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGAC TT-3'), PCR was performed using the extracted highly purified genomic DNA as a template under the following conditions: 95°C for 10 min, followed by 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s for 30 cycles with a final 10 min extension at 72°C. The PCR products were detected by agarose gel electrophoresis and then sent to GENEWIZ.lnc for the 16S rRNA gene sequencing. The phylogenetic status of the species was determined by a reaction of 700–750 bp (V1-V4) using the universal primers 27F, if the similarity was less than 98.65% (Kim et al. 2014), then the phylogenetic status of the species was further analyzed by nearly full-length 16S rRNA gene (1,300–1,400 bp).

Phylogenetic analysis. Similarity searches of the 16S rRNA gene sequences were performed in the NCBI and EzBiocloud database for BLAST, then the 16S rRNA gene sequences with the highest homology were obtained for phylogenetic analysis. The sequence alignments were performed using Clustal X, the phylogenetic trees were constructed from evolutionary distances using the neighbor-joining method with a bootstrap of 1,000 repetitions, and the phylogenetic analysis was conducted using the MEGA 7 software (Kumar et al. 2016b).

Nucleotide sequence accession numbers. The full and partial 16S rRNA gene sequences of the strains were submitted to the NCBI GenBank database under the accession numbers (MT611248 -MT611324).

Results

The isolated strains. Bacterial populations were successfully isolated from the highland barley cultivation soil sample using fifteen kinds of media, a total of 830 individual strains were obtained at different culture temperatures (4°C and 25°C) (Fig. 1A). Eighty-three and 747 strains of bacteria were isolated from these media at 4°C and 25°C, respectively. The results showed that X1, R, F1, M1, M5, M8, and GS culture media had a better effect on isolating bacteria at 25°C; however,

Table I
Isolation media.

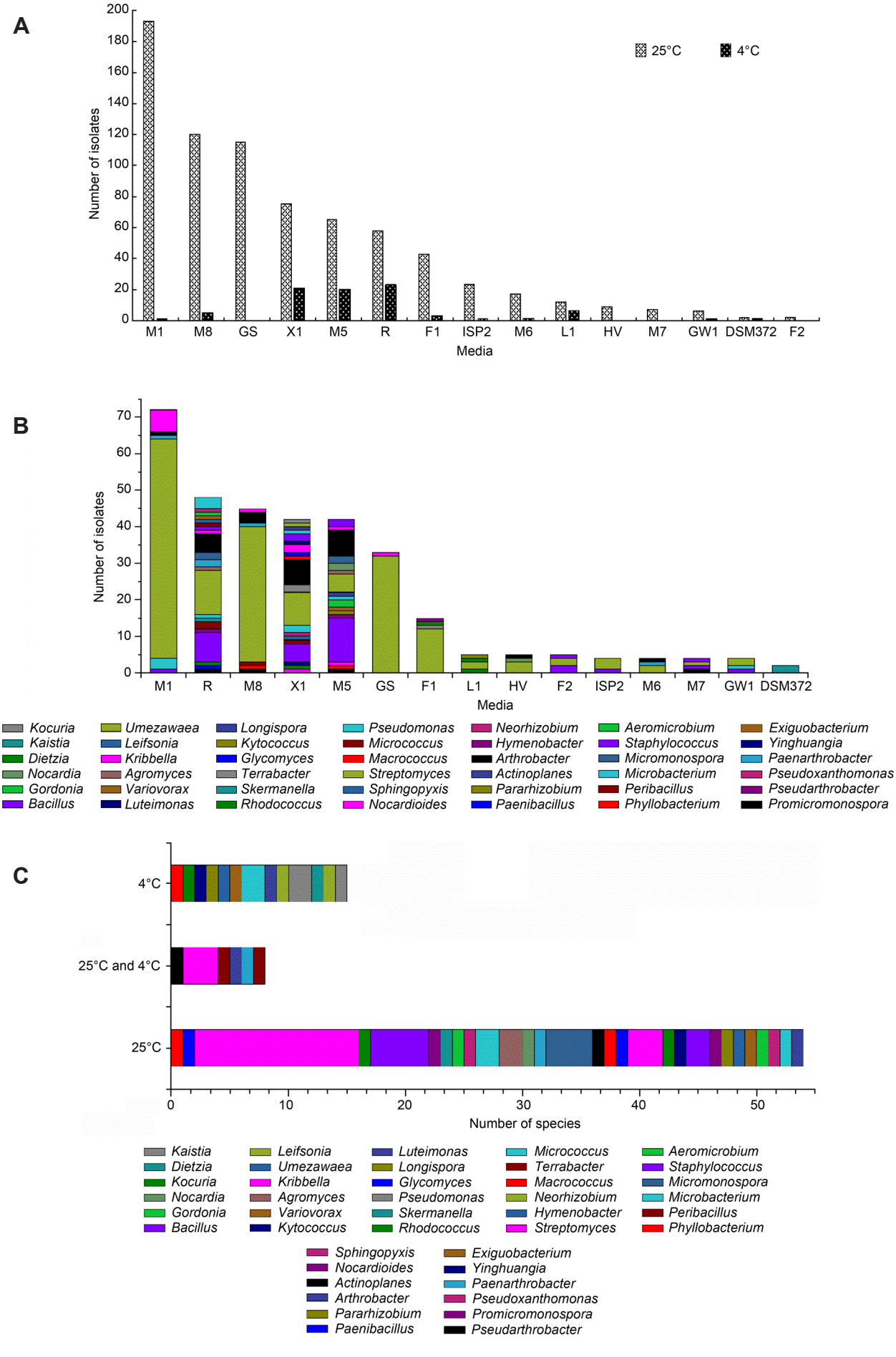
Media	Composition
X1	peptone 2.0 g, yeast extract 0.5 g, $FePO_4 \cdot 4H_2O$ 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 0.2 g, NaCl 0.5 g, agar 18.0 g, ddwater 1,000 ml, pH 7.0
R	peptone 10.0 g, yeast extract 5.0 g, maltose extract 5.0 g, casein amino acid 5.0 g, beef extract 2.0 g, glycerol 2.0 g, Tween-80 50.0 mg, $MgSO_4 \cdot 7H_2O$ 1.0 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.6
L1	NaCl 100.0 g, K_2HPO_4 5.0 g, $MgSO_4 \cdot 7H_2O$ 7.5 g, hydrolyzed casein 1.0 g, yeast extract 5.0 g, $Na_3C_6H_5O_7 \cdot 2H_2O$ 3.0 g, $FeSO_4 \cdot 7H_2O$ 0.1 g, $MnCl_2 \cdot 4H_2O$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 0.1 g, agar 18.0 g, ddwater 1,000 ml, pH 7.0–8.0
ISP2	NaCl 100.0 g, dextrose 4.0 g, yeast extract 4.0 g, maltose extract 10.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 2.0 g, $FeSO_4$ 10 mg, agar 18.0 g, ddwater 1,000 ml, pH 7.0–8.0
GW1	NaCl 100.0 g, casein 0.3 g, mannitol 1.0 g, $NaHCO_3$ 2.0 g, $CaCO_3$ 0.2 g, $(NH_4)_2SO_4$ 2.0 g, KNO_3 2.0 g, K_2HPO_4 1.0 g, $MgSO_4 \cdot 7H_2O$ 2.0 g, $FeSO_4$ 10.0 mg, Trace-salt 10.0 mg/l, Agar 18.0 g, ddwater 1,000 ml, pH natural
DSM372	NaCl 100.0 g, hydrolyzed casein 5.0 g, yeast extract 5.0 g, $Na_3C_6H_5O_7 \cdot 2H_2O$ 3.0 g, $Na_2CO_3 \cdot 10H_2O$ 8.0 g, $NaC_5H_8NO_4$ 1.0 g, KCl 2.0 g, $MgSO_4 \cdot 7H_2O$ 2.0 g, agar 18.0 g, ddwater 1,000 ml, pH natural
F1	glycerol 5.0 g, alanine 3.0 g, arginine 1.0 g, $(NH_4)_2SO_4$ 2.64 g, KH_2PO_4 2.38 g, K_2HPO_4 5.65 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, $CuSO_4 \cdot 5H_20$ 0.0064 g, $FeSO_4 \cdot 7H_2O$ 0.0011 g, $MnCl_2 \cdot 4H_2O$ 0.0079 g, $ZnSO_4 \cdot 7H_2O$ 0.0015 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin)
F2	$MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 0.2 g, $FeSO_4$ 10.0 mg, NaCl 0.5 g, $MnCl_2 \cdot 4H_2O$ 1.4 g, $Na_2MoO_4 \cdot 2H_2O$ 0.39 g, $Co(NO_3)_2 \cdot 6H_2O$ 0.025 g, $ZnSO_3 \cdot 7H_2O$ 0.222 g, $NaHCO_3$ 2.0 g, $NaH_2PO_4 \cdot 2H_2O$ 0.05 g, agar 18.0 g, ddwater 1,000 ml, pH natural (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin)
M1	soluble starch 10.0 g, casein 0.3 g, KNO_3 2.0 g, K_2HPO_4 2.0 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin)
M5	yeast extract 4.0 g, soluble starch 15.0 g, K_2HPO_4 1.0g, $FeSO_4 \cdot 7H_2O$ 0.01 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.6 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin)
M6	raffinose 10.0 g, L-histidine 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin)
M7	L-aspartic acid 0.1 g, peptone 2.0 g, sodium propionate 4.0 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin)
M8	glycerine 6.0 ml, arginine 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin)
HV	humic acid 1.0g, Na_2HPO_4 0.5 g, KCl 1.7 g, $MgSO_4$ 0.5 g, $FeSO_4$ 0.01 g, $CaCO_3$ 0.02 g, agar 18.0 g, ddwater 1,000 ml, pH 7.2–7.4 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin)
GS	soluble starch 20.0 g, NaCl 0.5 g, KNO_3 1.0 g, $K_2HPO_4 \cdot 3H_2O$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, agar 18.0 g, ddwater 1,000 ml, pH 7.4–7.6 (add 25 μg/ml nalidixic acid and 100 μg/ml nystatin)

X1, R, and M5 culture media had a better effect on isolating bacteria at 4°C, none of the bacteria was isolated from the F2, M7, HV, and GS media at 4°C.

Phylogenetic analysis of culturable strains by the 16S rRNA gene sequence. According to the morphological characteristics of bacteria, 330 strains were screened for the 16S rRNA gene sequence analysis using the universal primers 27F/1492R, and 98.65% of the 16S rRNA gene sequences were used as the species boundary of prokaryotes. After combining more than 98.65% of the 16S rRNA gene sequences with the same species, the sequences of 77 species were obtained, which belonged to 42 genera and four phyla (Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes), as shown in Table II. Phylogenetic tree based on the 16S rRNA gene sequences of representative bacteria strains were shown (Fig. 2).

There were 53 species and 25 genera in Actinobacteria, accounting for 68.82% of the species' total number. The predominant genus was *Streptomyces* (22.08%, 17 species), followed by *Micromonospora* (5.19%, four species), *Microbacterium* (5.19%, four species), and *Kribbella* (3.90%, three species). Some rare Actinobacteria were also isolated, for example, *Leifsonia*, *Longispora*, *Nocardia*, *Nocardioides*, *Terrabacter*, *Umezawaea,* and *Kribbella*. There were 12 species and ten genera in Proteobacteria, accounting for 15.59% of the total number of species, but no dominant genus was found in Proteobacteria. There were 11 species and six genera in Firmicutes, accounting for 14.29% of the total number of species; the predominant genus was *Bacillus* (6.49%, five species). Only one species was found in Bacteroidetes, classified as *Hymenobacter* (1.30%, one species) (Table III).

Diversity of culturable strains recovered from different culture media. Among the 330 identified bacteria strains, the number of bacterial isolates recovered from M1 was the largest (21.82%, 72 strains), followed


A
25°C
4°C
Number of isolates
Media
M1 M8 GS X1 M5 R F1 ISP2 M6 L1 HV M7 GW1 DSM372 F2
B
Number of isolates
Media
M1 R M8 X1 M5 GS F1 L1 HV F2 ISP2 M6 M7 GW1 DSM372
Kocuria Umezawaea Longispora Pseudomonas Neorhizobium Aeromicrobium Exiguobacterium
Kaistia Leifsonia Kytococcus Micrococcus Hymenobacter Staphylococcus Yinghuangia
Dietzia Kribbella Glycomyces Macrococcus Arthrobacter Micromonospora Paenarthrobacter
Nocardia Agromyces Terrabacter Streptomyces Actinoplanes Microbacterium Pseudoxanthomonas
Gordonia Variovorax Skermanella Sphingopyxis Pararhizobium Peribacillus Pseudarthrobacter
Bacillus Luteimonas Rhodococcus Nocardioides Paenibacillus Phyllobacterium Promicromonospora
C
4°C
25°C and 4°C
25°C
Number of species
Kaistia Leifsonia Luteimonas Micrococcus Aeromicrobium
Dietzia Umezawaea Longispora Terrabacter Staphylococcus
Kocuria Kribbella Glycomyces Macrococcus Micromonospora
Nocardia Agromyces Pseudomonas Neorhizobium Microbacterium
Gordonia Variovorax Skermanella Hymenobacter Peribacillus
Bacillus Kytococcus Rhodococcus Streptomyces Phyllobacterium
Sphingopyxis Exiguobacterium
Nocardioides Yinghuangia
Actinoplanes Paenarthrobacter
Arthrobacter Pseudoxanthomonas
Pararhizobium Promicromonospora
Paenibacillus Pseudarthrobacter

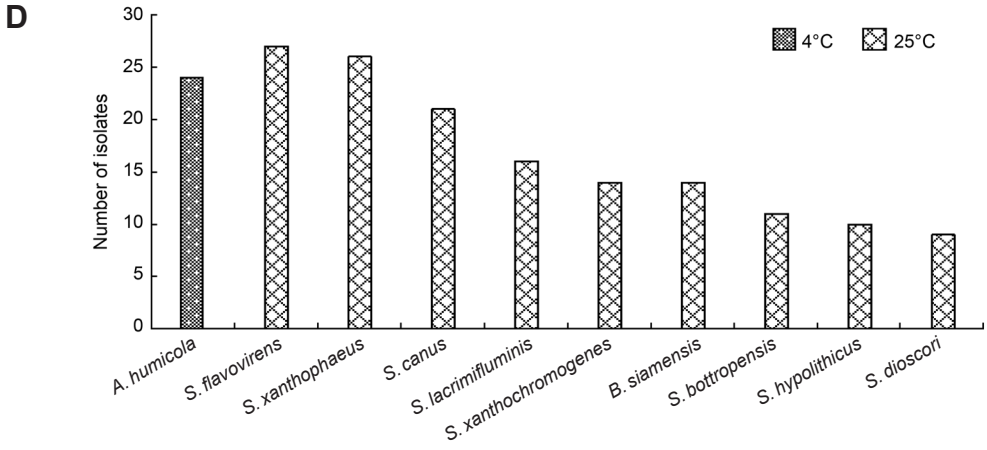


Fig. 1. The number and diversity of bacteria.

A) The numbers of bacteria isolated from different media at 4° and 25°. B) Diversity of bacteria isolated from different culture media. C) Diversity of bacteria isolated from different temperature. D) The numbers of dominant species isolated from 4° and 25°.

by R (14.55%, 48 strains), M8 (13.64%, 45 strains), X1 (12.73%, 42 strains), M5 (12.73%, 42 strains), GS (10.00%, 33 strains), F1 (4.55%, 15 strains), L1 (1.52%, five strains), F2 (1.52%, five strains), HV (1.52%, five strains), ISPT2 (1.21%, four strains), M6 (1.21%, four strains), M7 (1.21%, four strains), GW1 (1.21%, four strains), and DSM372 (0.61%, two strains). The number of bacteria isolated from M1, M8, and GS was larger, while the main genus was only *Streptomyces*. The R, X1, and M5 yielded higher genera diversity (21 genera, 20 genera, and 17 genera, respectively). Meanwhile, media R, X1, and M5 were more useful than other media for isolation of rare genera of bacteria, such as *Nocardioides*, *Leifsonia*, *Terrabacte*, *Umezawaea*, *Variovorax*, *Neorhizobium,* and *Pararhizobium* (Fig. 1B). Here, we presumed that single-nutrition was the main reason, especially when non-monosaccharide was used as the carbon source (Zhang et al. 2010b; Kurm et al. 2019). This study demonstrated that it is necessary to use various isolation media types to increase the number and diversity of bacteria from highland barley cultivation soil samples.

Diversity of culturable strains at different temperature. There were 62 species and 33 genera bacteria isolated at 25°C, accounting for 80.52% of the species' total number. The predominant genus was *Streptomyces* (22.08%, 17 species), followed by *Bacillus* (6.49%, five species), *Micromonospora* (5.19%, four species), *Kribbella* (3.90%, three species), and *Paenarthrobacter* (3.90%, three species). There were 23 species and 18 genera bacteria isolated at 4°C, accounting for 29.87% of the total species, but no dominant genus was found. Meanwhile, only eight species and six genera of bacteria could be isolated at 25°C and 4°C (Fig. 1C). Most common bacteria could be isolated at 25°C, but some rare bacteria could be isolated at 4°C without

Table II
Genera distributed in each of the four phyla.

Actinobacteria		Proteobacteria	Firmicutes	Bacteroidetes
Actinoplanes	*Micrococcus*	*Kaistia*	*Bacillus*	*Hymenobacter*
Aeromicrobium	*Micromonospora*	*Luteimonas*	*Exiguobacterium*	
Agromyces	*Nocardia*	*Neorhizobium*	*Macrococcus*	
Arthrobacter	*Nocardioides*	*Pararhizobium*	*Paenibacillus*	
Dietzia	*Paenarthrobacter*	*Phyllobacterium*	*Peribacillus*	
Glycomyces	*Promicromonospora*	*Pseudomonas*	*Staphylococcus*	
Gordonia	*Pseudarthrobacter*	*Pseudoxanthomonas*		
Kocuria	*Rhodococcus*	*Skermanella*		
Kribbella	*Streptomyces*	*Sphingopyxis*		
Kytococcus	*Terrabacter*	*Variovorax*		
Leifsonia	*Umezawaea*			
Longispora	*Yinghuangia*			
Microbacterium				

Table III
BLAST results based on 16S rRNA gene sequences of 77 bacterial species.

Strain number	Name of strain having the highest 16S rRNA gene similarity	The highest similarity (%)	Strain number	Name of strain having the highest 16S rRNA gene similarity	The highest similarity (%)
T74*	*Actinoplanes digitatis* IFO 12512	98.82	T608	*Paenarthrobacter aurescens* NBRC 12136	99.07
T203	*Aeromicrobium ginsengisoli* Gsoil 098	99.82	T236	*Paenarthrobacter nitroguajacolicus* G2-1	100
T96*	*Agromyces binzhouensis* OAct353	98.62	T808*	*Pararhizobium herbae* CCBAU 83011	98.79
T229*	*Agromyces humatus* CD5	98.74	T209	*Peribacillus simplex* NBRC 15720	100
T805	*Arthrobacter crystallopoietes* DSM 20117	99.85	T811	*Phyllobacterium ifriqiyense* STM 370	100
T763	*Arthrobacter humicola* KV-653	100	T274*	*Phyllobacterium zundukense* Tri-48	98.57
T65	*Bacillus siamensis* KCTC 13613	100	T63	*Promicromonospora alba* 1C-HV12	100
T94	*Bacillus cereus* ATCC 14579	100	T193*	*Pseudarthrobacter siccitolerans* 4J27	99.34
T228*	*Bacillus drentensis* LMG 21831	99.34	T755	*Pseudomonas laurylsulfativorans* AP3_22	99.73
T59	*Bacillus pumilus* ATCC 7061	100	T776	*Pseudomonas lini* CFBP 5737	100
T115	B*acillus selenatarsenatis* SF-1	99.6	T174*	*Pseudoxanthomonas sacheonensis* BD-c54	99.34
T822	*Dietzia kunjamensis* subsp DSM 44907	99.86	T127*	*Rhodococcus jostii* DSM 44719	99.32
T230	*Exiguobacterium mexicanum* 8NT	100	T788	*Rhodococcus qingshengii* JCM 15477	100
T183*	*Glycomyces algeriensis* NRRL B-16327	98.9	T185*	*Skermanella aerolata* 5416T-32	98.86
T64	*Gordonia otitidis* NBRC 100426	100	T93	*Sphingopyxis fribergensis* Kp5.2	99.87
T830*	*Hymenobacter humi* DG31A	98.60	T45	*Staphylococcus caprae* ATCC 35538	100
T769*	*Kaistia defluvii* B6-12	99.72	T61	*Staphylococcus cohnii* subsp ATCC 49330	100
T144	*Kocuria sediminis* FCS-11	99.43	T666	*Streptomyces albogriseolus* NRRL B-1305	100
T145	*Kribbella albertanoniae* BC640	100	T313	*Streptomyces atroolivaceus* NRRL ISP-5137	100
T214*	*Kribbella catacumbae* DSM 19601	99.6	T234	*Streptomyces bottropensis* ATCC 25435	99.87
T422	*Kribbella karoonensis* Q41	99.87	T130	*Streptomyces caniferus* NBRC 15389	99.87
T823	*Kytococcus schroeteri* DSM 13884	99.73	T235	*Streptomyces canus* DSM 40017	99.73
T781	*Leifsonia flava* SYP-B2174	99.73	T690	*Streptomyces dioscori* A217	99.47
T146	*Longispora urticae* NEAU-PCY-3	99.88	T532	*Streptomyces flavovirens* NBRC 3716	99.85
T181*	*Luteimonas composti* CC-YY255	98.9	T674*	*Streptomyces humidus* NBRC 12877	98.8
T156	*Macrococcus canis* KM 45013	99.86	T296	*Streptomyces hydrogenans* NBRC 13475	99.46
T489	*Microbacterium maritypicum* DSM 12512	99.55	T219	*Streptomyces hypolithicus* HSM10	99.46
T773	*Microbacterium natoriense* TNJL143-2	99.87	T426	*Streptomyces kurssanovii* NBRC 13192	99.6
T804	*Microbacterium phyllosphaerae* DSM 13468	99.73	T569	*Streptomyces lunaelactis* MM109	99.2
T133	*Microbacterium thalassium* IFO 16060	98.93	T348	*Streptomyces niveus* NRRL 2466	99.46
T226	*Micrococcus luteus* NCTC 2665	99.63	T84	*Streptomyces phaeoluteigriseus* DSM 41896	99.6
T47	*Micromonospora cremea* DSM 45599	99.87	T581	*Streptomyces turgidiscabies* ATCC 700248	100
T206	*Micromonospora luteifusca* GUI2	99.87	T110*	*Streptomyces xanthochromogenes* NRRL B-5410	98.97
T197*	*Micromonospora palomenae* NEAU-CX1	98.74	T100	*Streptomyces xanthophaeus* NRRL B-5414	99.71
T92	*Micromonospora saelicesensis* Lupac 09	100	T111*	*Terrabacter ginsengisoli* Gsoil 653	99.19
T786*	*Neorhizobium vignae* CCBAU 05176	98.70	T160	*Umezawaea tangerina* NRRL B-24463	99.18
T62	*Nocardia salmonicida* subsp R89	99.47	T812	*Variovorax boronicumulans* BAM-48	99.47
T105*	*Nocardioides caeni* MN8	98.01	T134*	*Yinghuangia seranimata* YIM 45720	98.73
T218	*Paenibacillus odorifer* DSM 15391	99.63			

* – shown that the full length 16S rRNA gene of this bacterium was sequenced

the inhibitory effect of dominant species, promoting the diversity of bacteria (Margesin 2012; Collins and Margesin 2019). The numbers of dominant species mainly isolated at 4°C were *Arthrobacter humicola* (7.25%, 24 strains), while the numbers of dominant species mainly isolated at 25°C were *Streptomyces flavovirens* (8.19%, 27 strains), *Streptomyces xanthophaeus* (7.58%, 25 strains), *Streptomyces canus* (6.36%, 21 strains), and *Bacillus siamensis* (4.24%, 14 strains) (Fig. 1D). The species of culturable bacteria and the

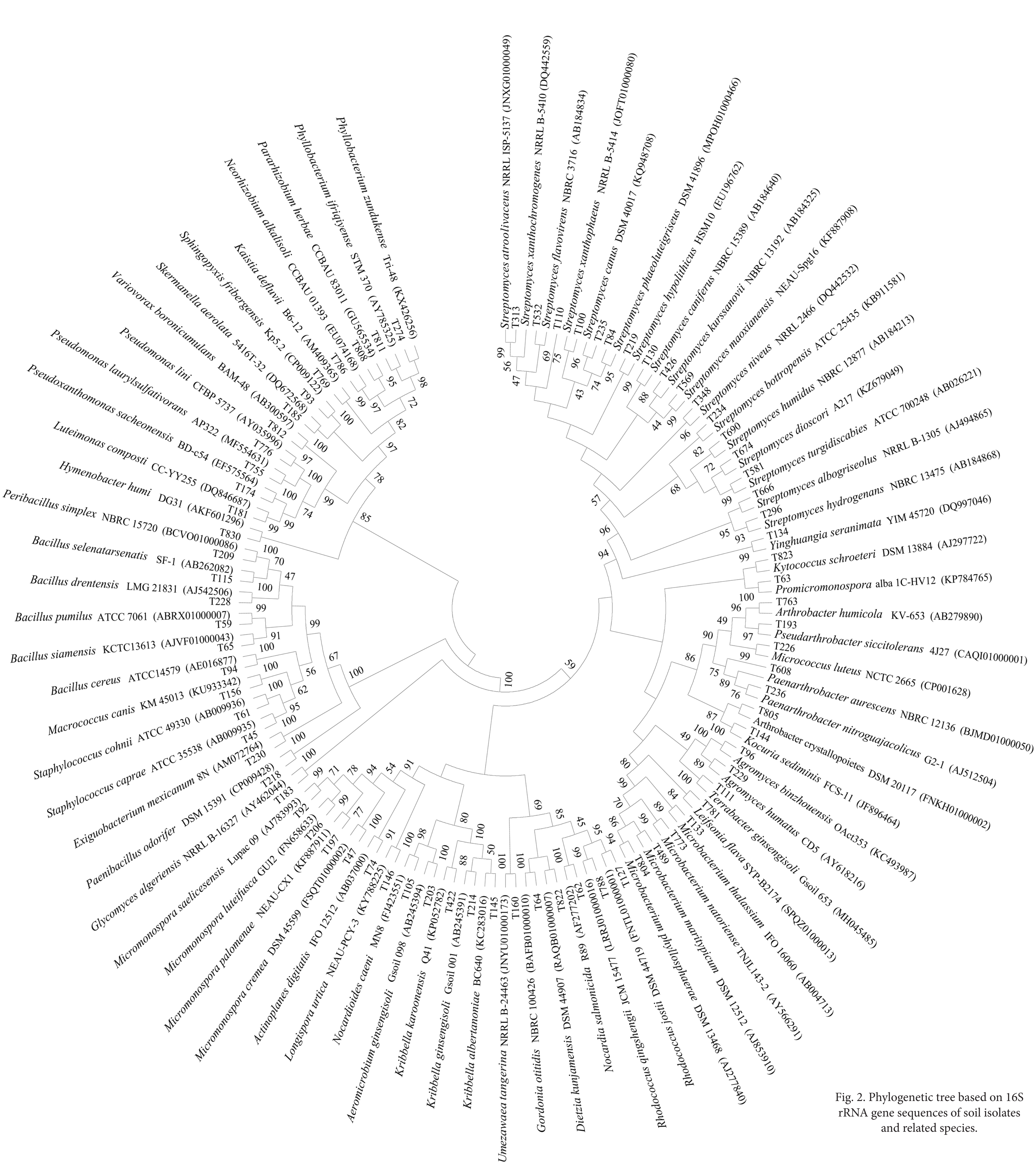


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of soil isolates and related species.

Table IV
The sequence analyses based on almost full-length of the 16S rRNA gene of six potential new species.

Strain number	Name of strain having the highest 16S rRNA gene similarity	Separation medium	The highest similarity (%)	Separation temperature (°C)
T96	*Agromyces binzhouensis* OAct353^T	98.62	M5	25
T105	*Nocardioides caeni* MN8^T	98.01	M5	25
T274	*Phyllobacterium zundukense* Tri-48^T	98.57	M8	25
T786	*Neorhizobium vignae* CCBAU 05176^T	98.70	R	4
T808	*Pararhizobium herbae* CCBAU 83011^T	98.79	M5	4
T830	*Hymenobacter humi* DG31A^T	98.60	F1	4

numbers of dominant species were significantly different at 4°C and 25°C in this study.

Potential new species information. Among the 77 species, four bacterial strains exhibited low 16S rRNA gene sequence similarities (< 98.65 %) with validly described species based on the results of the BLAST search in EzBiocloud (Table IV), which indicated that these isolates could represent novel taxa. *Neorhizobium* gen. nov. was a new genus of rhizobia established by Mousavi et al. (2014); so far, only five species had been published. The T786 strain had 98.70%, 98.47%, 98.24%, 98.16%, 97.79%, and 96.55% sequence similarity with *Neorhizobium vignae* CCBAU 05176^T (GU128881), *Neorhizobium alkalisoli* CCBAU01393^T (EU074168), *Neorhizobium tomejilense* T17_20^T (PVBG01000052), *Neorhizobium huautlense* S02^T (AF025852), *Neorhizobium galegae* ATCC43677T(D11343), and *Neorhizobium lilium* 24NR^T (MK386721), respectively (Fig. 3). Further data analysis suggested that the dDDH and ANI values between strain T786 and *N. vignae* CCBAU 05176^T, *N. alkalisoli* CCBAU 01393^T, *N. tomejilense* T17_20^T, *N. huautlense* S02^T, and *N. galegae* ATCC 43677^T were 20.20–20.50% and 76.64–80.01%, respectively, which were lower than the threshold values of 70% and 95–96% for species discrimination (unpublished). *Pararhizobium* gen. nov. was a new genus of rhizobia also established by Mousavi et al. (2015); so far, only seven species had been published. The T808 strain had high similarity with *Pararhizobium herbae* CCBAU83011^T (GU565534) (98.79%), *Pararhizobium polonicum* F5.1^T (LGLV01000030) (98.65%), and *Pararhizobium giardinii* H152^T (ARBG01000149) (98.50%). The 16S rRNA sequence of strain T808 had about 40 more bases in the V1-V2 region than the seven validly published species of *Pararhizobium*, while the NCBI database showed that T808 had 98.54–99.15% similarity with Uncultured bacterium clone barrow_FF_26 (JX668750.1), *Rhizobium* sp.Ia8 (KF444807), and Rhizobiaceae bacterium strain FW305-C-27(MN067584), all of which were uncultured bacteria without lacking the 40 bases in V1-V2 region (Fig. 4). Based on the above analysis, T808 might be a potentially new species of *Pararhizobium*. *Neorhizobium* and *Pararhizobium* were important non-symbiotic species of rhizobia with poor nodulation or nitrogen fixation genes, which have the important microbial niche value (Shen et al. 2018; Soenens et al. 2019).

Three potential new species were isolated from media M5, and one species was isolated from media M8, R, and F1, respectively. Half of six potential new species were cultured at 4°C, while others were cultured at 25°C. The culture medium and temperature have a significant influence on the separation of new species. All six potential new species will be further identified with a polyphasic approach (including chemotaxonomic properties, DNA-DNA hybridization analysis) to determine their taxonomic positions.

Discussion

Together with the incubation of the highland barley cultivation soil sample using fifteen kinds of media at 25°C and 4°C, a total of 830 individual strains were purified. The 16S rRNA gene sequence analysis results are consistent with a previous report, in which Actinobacteria, Proteobacteria, Firmicutes were found to be dominant phyla in the arctic-alpine area, especially in the Qinghai-Tibet plateau (Jiang et al. 2006; Kumar et al. 2016a; Tang et al. 2016). The predominant genus was *Streptomyces*, followed by *Bacillus*, *Micromonospora*, and *Microbacterium*. The most diverse isolates belonged to high the G+C Gram-positive group; in particular, the *Streptomyces* genus is a dominant genus in the high G+C Gram-positive group. The bacteria in arctic-alpine areas are mainly the spore producing, stress-resistant, and thick cell walls microorganisms (Zhang et al. 2010b; Rao et al. 2016).

The Actinobacteria are widely dispersed throughout the highland barley cultivation soil, while few studies are on it. The bacteria in highland barley cultivation soil in Lhasa analyzed by high-throughput sequencing technology showed that the main actinomycetes were *Gaiiella*, *Arthrobacter*, and *Nocardioides* (Liu et al.

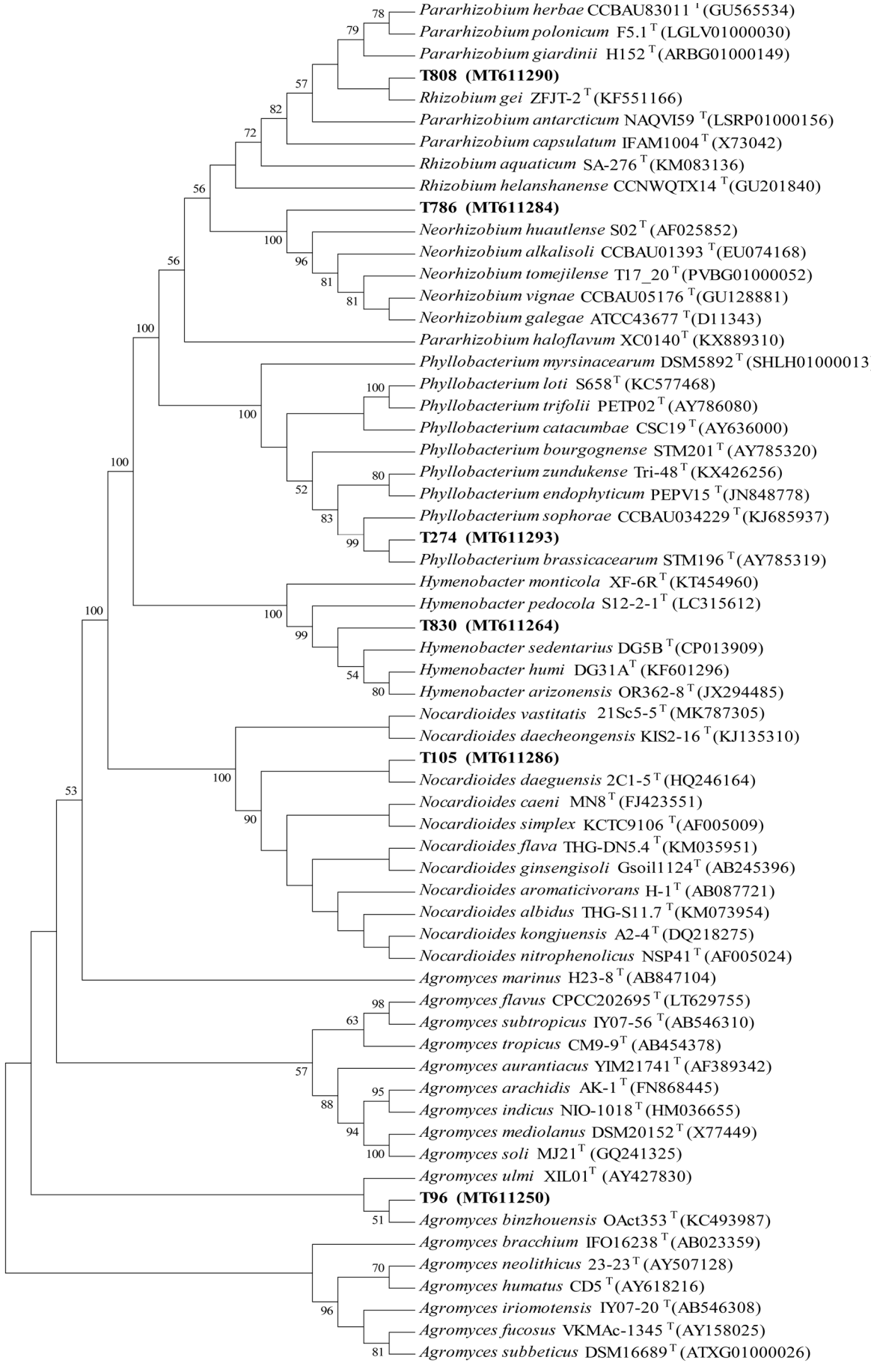


Fig. 3. Phylogenetic tree based on the16S rRNA gene sequences of new candidates and related species.

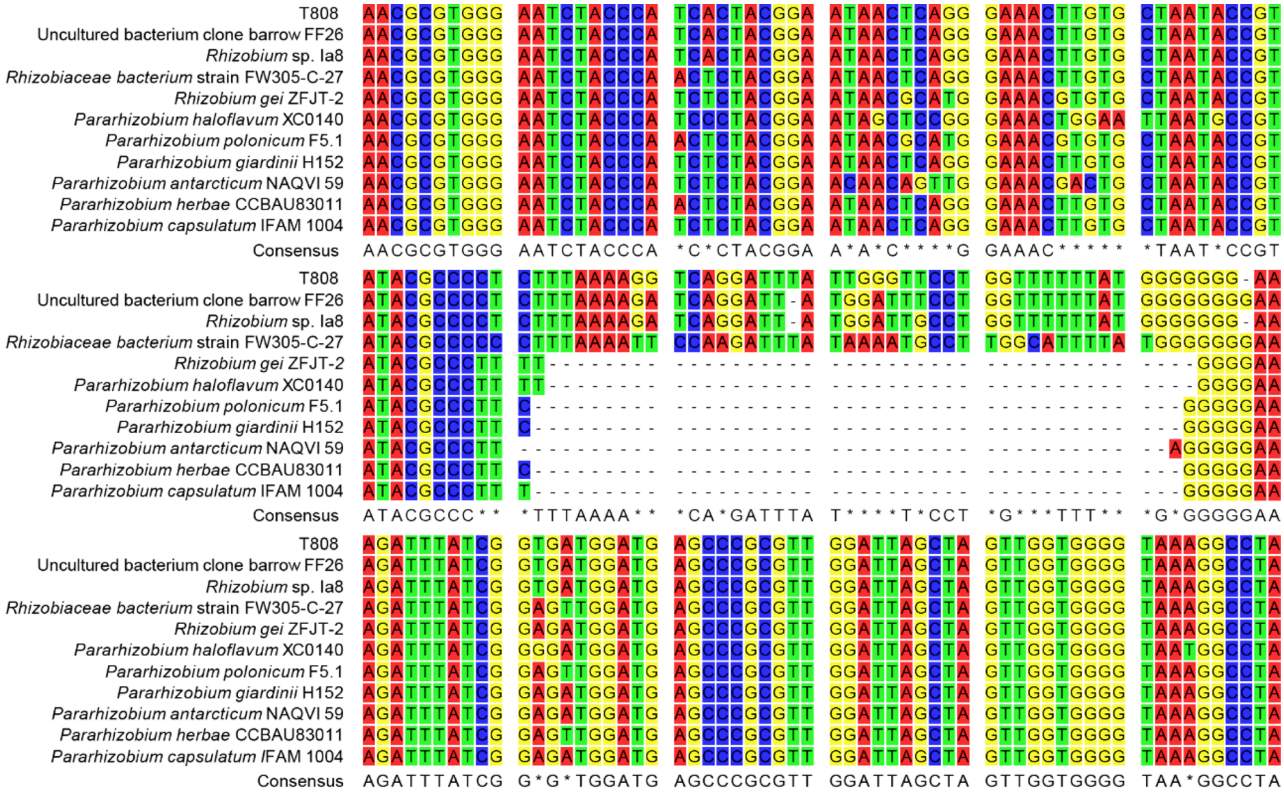


Fig. 4. The Clustal X analysis of strain T808.

2020), which was quite different from our study using the culturable technique. In other previous reports, the main genus in the highland barley cultivation soil was *Streptomyces*, *Arthrobacter*, and *Nocardioides*. Most Actinomycetes had a wide spectrum of inhibitory activity against pathogenic bacteria, highly IAA production, and phosphate solubilization, which were in similarity with our study (Qi et al. 2017; Yin et al. 2017; Gao et al. 2019). As the most well-known genus in Actinobacteria, *Streptomyces* contains 960 species (http://www.bacterio.net/streptomyces) and 4227 genome assemblies available (https://www.ncbi.nlm.nih.gov/genome/streptomyces) at the time of writing. Members of the genus *Streptomyces* are well known as the primary sources of antibiotics with diverse biological activities and chemical structures (Jones and Elliot 2017; Li et al. 2018). In this study, 17 species of *Streptomyces* were found in the highland barley cultivation soil, the larger numbers of dominant species of *Streptomyces* were *Streptomyces flavovirens*, *Streptomyces xanthophaeus* and *Streptomyces canus*, which were mainly isolated at 25°C. The Qamdo region's temperature is between 20°C and 28°C from June to September, which is also a critical growth period for highland barley. We believe that these *Streptomyces* that can produce many biological activities have an essential role in the growth of highland barley in this period. The other dominant isolates in highland barley cultivation soil were *Arthrobacter humicola* and *Bacillus siamensis*, which are important plant growth-promoting rhizobacteria (PGPR) (Bai et al. 2015).

Meanwhile, *Arthrobacter humicola* was mainly isolated at 4°C, producing cold lipase and biopolymeric flocculant (Agunbiade et al. 2017). The low-temperature adaptation and ecological function of *A. humicola* in highland barley cultivation soil need to be studied in-depth. Some rare Actinobacteria were also isolated from the soil sample, for example, *Leifsonia*, *Longispora*, *Nocardia*, *Nocardioides*, *Terrabacter*, *Umezawaea*, and *Kribbella*. Rare Actinobacteria are also important sources in discovering novel antibiotics and have been seldom studied (Cai et al. 2018; Bundale et al. 2019).

In summary, this study has demonstrated a rich diversity of bacteria (especially Actinobacteria) and some undiscovered bacteria species in the highland barley cultivation soil of Qinghai-Tibet plateau it suggests that these strains might represent a valuable source of new taxa for further microbial development and utilization. Additionally, this study indicates that cultivating Actinobacteria in highland barley cultivation soil of Qinghai-Tibet plateau could be interesting for further study.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication

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