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NOTE

Diversity analysis of biocontrol *Bacillus* isolated from rhizospheric soil of rice–wheat (*Oryza sativa–Triticum aestivum* L.) at India

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Future food situation will be strongly dominated by the changes in the population structures, developing economies, changes in diet and associated demand for food.[1] It is assumed that the growth scenario of the population is predicated to increase by a further 700 million people in the Indogangetic Plains of India (IGP) (about equal to the current population of Europe) in the next 30 years. This will result in a greater demand for food, and it is estimated that the food grain requirement by 2020 in the region will be almost 50% more than at present.[2] The IGP is now called as the 'bread basket' for South Asia, which is known to be the most extensive fluvial plains of the world. Rice and wheat are the two major cereal crops of this region, and this region produces about 50% of the total foodgrains to feed 40% of country's population.[3] IGP crops were consistently infested with endemic diseases such as sheath, rots, and wilt diseases caused by phytopathogenic fungi, that is, *Rhizoctonia*, *Macrophomina* and *Fusarium* were difficult to control because of their soil-borne pathogenic nature. They affect countries economy (US$36 million losses in India), as well as crop yields in tropical and subtropical regions.[4,5] Pesticides were reported to cause adverse effects on soil ecosystem and also induce resistance in pathogens.[6] Nowadays biological pesticides are used as a potential tool to replace or augment conventional plant disease management that makes use of synthetic pesticides.[7–10] Rhizobacteria are beneficial to crops yields via nutrient acquisition,[11] biocontrol,[12,13] plant hormone-like production[14] and induction of systemic resistance.[15] *Bacillus* is cosmopolitan in nature contributing 92% soil microbial library and effective biocontrol agent for various pests such as *Macrophomina phaseolina*,[16,17] *Rhizoctonia solani*[18,19] and *Fusarium udum*.[20,21] Various *Bacillus* species have been reported for their capacities to protect plants from pathogens and stimulate plant growth since long time.[16–19] They are used as a suitable biocontrol agent because of their ability to grow rapidly *in vivo* as well as *in vitro* condition, colonize and multiply in the rhizosphere, and compete aggressively with other microorganisms.[22] The estimation of microbial diversity along with study of its ecological habitat is required for understanding its biogeography, community assembly and ecological processes.[23] Ribosomal genes (16S rRNA gene) sequencing provides sufficient genetic information for bacterial identification, and it has been widely applied to the identification of different *Bacillus* species.[24,25] Amplified rDNA restriction analysis (ARDRA) has been useful for the identification and de-duplication of the microbes within a genus as well as at species level.[26] In the present work, we discussed the population frequency and diversity analysis of *Bacillus* showing antagonisms against endemic tropical and subtemperate soil-borne phytopathogens (*M. phaseolina*, *R. solani* and *F. udum*), from different regions of IGP, India, as well as analysis of soil macronutrient composition (OC, N, P and K) and molecular characterization of *Bacillus* isolates through ARDRA analysis followed by identification by 16S rRNA gene sequencing of antagonistic *Bacillus* from IGP regions of India (see Supplementary Information).

The study area covered a total of 55 rhizospheric soil samples (wheat–rice cropping system) from different IGP regions, India, by using random stratified sampling techniques. The selected IGP regions included six states with coordinates such as Uttrakhand (30°19′48″- 30°33′N, 78°3′36″- 78°06′E), Uttar Pradesh (26°51′0″- 26°85′N, 80°54′36″- 80°91′E), Haryana (30°43′48″- 30°73′N, 76°46′48″- 76°78′E), Punjab (30°47′24″-30°79′N, 76°46′48″- 76°78′E), Rajgir (Bihar) (25°1′48″- 25°03′N, 85°25′12″- 85°42′E) and West Bengal states (22°34′0″- 22°566667′N, 88°22′0″- 88°366667′E) (Figure 1). Sediment samples were collected at 10–12 days intervals from each site in triplicates. Rhizospheric soil monolith of 5 cm^3 along with root were randomly removed from the wheat–rice crop field system, aseptically stored in sterile polythene bags, brought to the laboratory and dried at 30 °C to obtain constant weight. Each composite soil sample was divided into two parts. One part in the field moist condition was used for determination of soil physiochemical parameters, while other part was used for *Bacillus* isolation. Samples were kept at 4 °C for further analysis. Soil texture and

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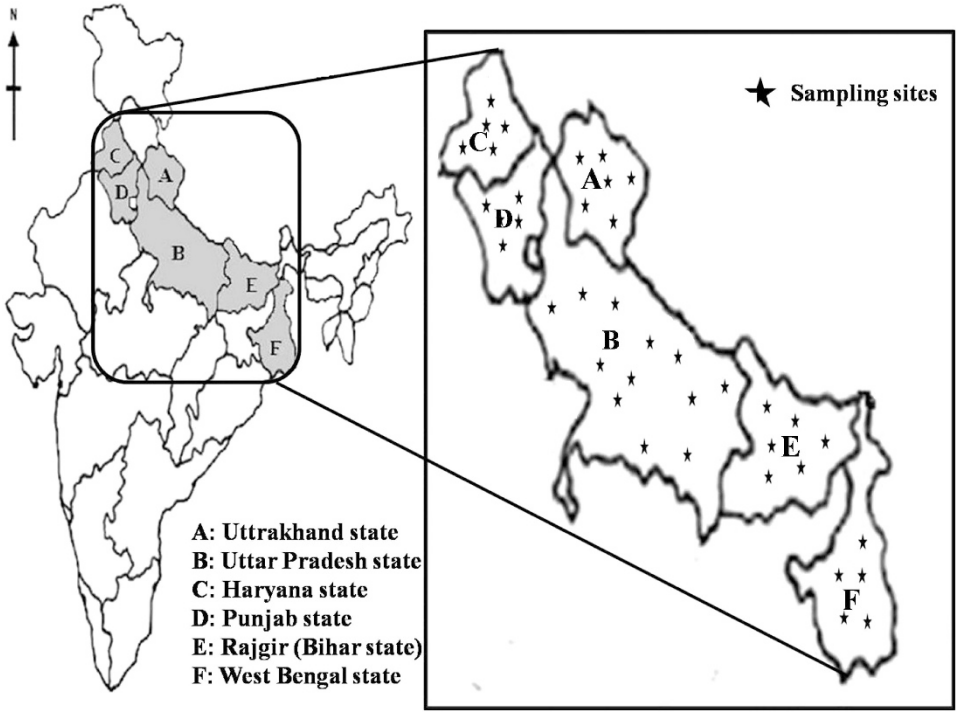

Figure 1 Map showing the sites of soil sample collection ((A) Uttrakhand; (B) Uttar Pradesh; (C) Haryana; (D) Punjab; (E) Rajgir and (F) West Bengal states) from Indo-Gangetic Plain, India.

Table 1 Geographical location, soil physiochemical properties and enumeration of *Bacillus* isolates from Indogangetic Plains of India

Location	*Coordinates*	*CFU* $\times 10^4$ *per g of soil*	*Soil texture*	*pH*	*EC*	*N* ($kg\,ha^{-1}$)	*P* ($kg\,ha^{-1}$)	*K* ($kg\,ha^{-1}$)	*Total isolates picked*	*Biocontrol*[a] *Bacillus isolates (%)*
Haridwar	30°33′N 78°06′E	6–35	Loamy–sandy loam	6.91	0.14	145.5	28.8	70.2	31	17 (54.84)
Dehardun	29°23′N 79°27′E	3–32	Loamy–sandy loam	8.17	0.3	65.2	70.8	278.5	30	15 (50.00)
Nanital	29°96′N 78°16′E	8–24	Loamy–sandy loam	8.5	0.32	45.2	45.2	296.4	21	14 (66.67)
Almora	29°59′N 79°65′E	3–15	Silt loam	7.21	0.15	180.1	20.6	105.5	13	9 (69.24)
Haryana	30°79′N 76°78′E	8–42	Clay–silt loam	7.1	0.08	110.9	35.9	79.6	40	12 (30.00)
Punjab	27°37′N 74° 28′E	17–68	Clay–silt loam	7.16	0.14	132.9	64.7	55.1	64	18 (28.13)
Lucknow	26°51′N 80°55′E	13–72	Silt clay loam	9.83	1.1	70.2	46.2	395.1	60	20 (33.34)
Rajgir	25.03°N 85.42°E	10–46	Silt–clay loam	9.17	0.5	108.9	27.7	360.2	31	20 (64.52)
West Bengal	22°56′N 77°42′E	16–49	Loam–clay loam	8.32	0.33	52.7	46.4	272.6	44	12 (27.28)

Abbreviations: EC, electrical conductivity; N, nitrogen; K, potassium; OC, organic carbon; P, phosphorous.
Haridwar, Dehardun, Nanital and Almora are located in Uttrakhand state. Lucknow and Rajgir are located in Uttar Pradesh and Bihar states, respectively.
[a]Data in triplicates (positive assay against single/multiple phytopathogenic fungus).

physiochemical properties of soil samples were characterized according to the standard methods.[27]

A total of three hundred thirty-four *Bacillus* isolates were isolated from different ecoregions of IGP. The population frequencies of *Bacillus* isolates were fluctuated ($3–72 \times 10^4$ g soil $^{-1}$) such as Punjab belt soils harboured maximum *Bacillus* population while, Uttrakhand region had least population counts (Table 1). The fluctuation of *Bacillus* distribution may be due to difference in the physiochemical properties of soil and non uniform distribution of nutrients also.[28]

Screening of *Bacillus* isolates for the antimicrobial assay against the three test phytopathogenic fungi (*M. phaseolina, F. udum* and *R. solani*) revealed that a total of 41% isolates showed antagonism against test phytopathogens, whereas, 19.1, 13.1 and 9.5% isolates showed inhibition zone towards individually tested pathogens (that is, *M. phaseolina, F. udum* and *R. solani*). Moreover, screening based on inhibition zone revealed that a total of 9.2, 5.7 and 3.5% isolates showed more promising (zone of inhibition (ZOI) > 15 mm) towards *M. phaseolina, F. udum* and *R. solani*, respectively. Further clustering of isolates were carried out by various combination of phytopathogenic fungal inhibition parameter (ZOI > 15 mm), that is, 1.4% *Bacillus* isolates showed inhibition to all test pathogens (*M. phaseolina, F. udum* and *R. solani*), followed by combination of two test fungi, that is, *M. phaseolina*/*F. udum* (3.5%), *M. phaseolina*/*R. solani* (2.8%) and lastly *F. udum*/*R. solani* (2.1%), respectively. The distribution of biocontrol-based *Bacillus* isolates showed that Uttrakhand regions had maximum biocontrol potential isolates followed by

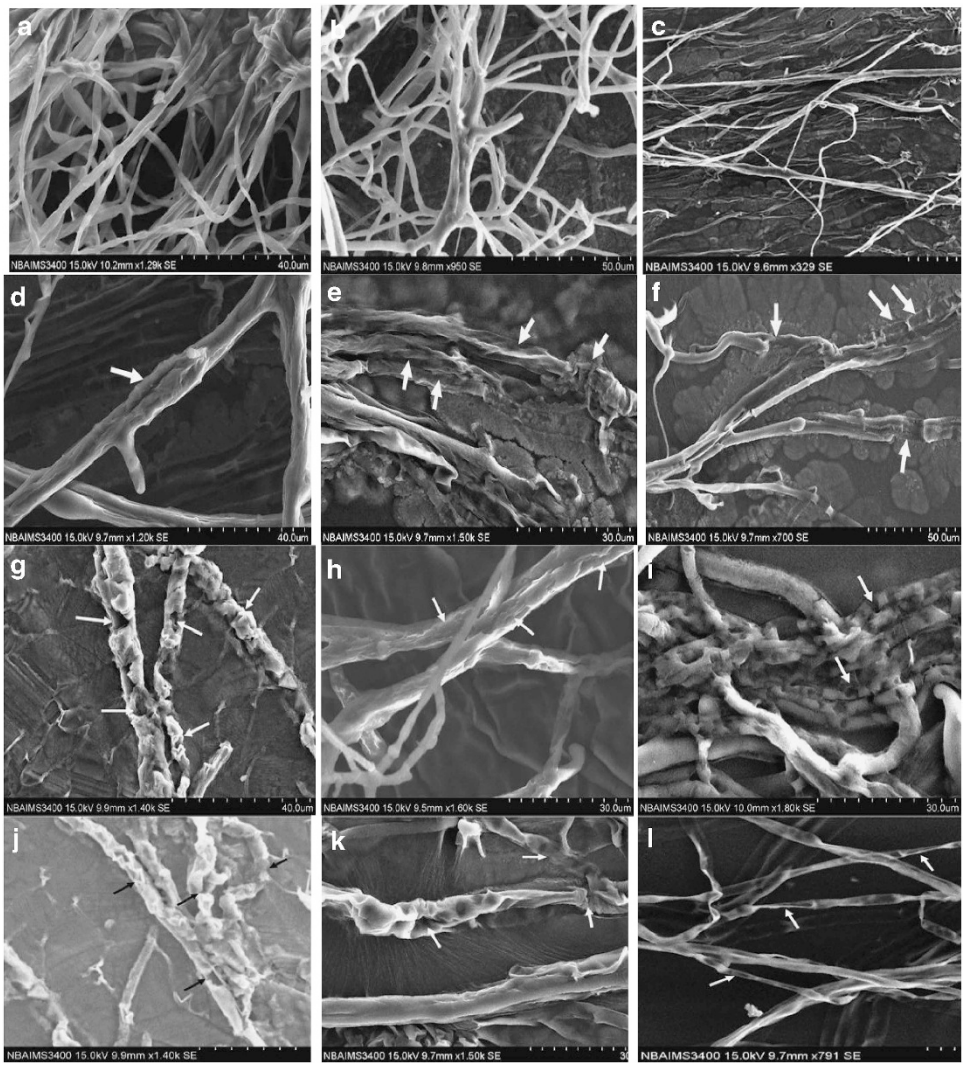

Figure 2 Scanning electron microscopy (SEM) of antagonist *Bacillus* isolates (TN10) from IGP, India (arrows indicates abnormal growth of fungal mycelia). Healthy mycelia were used as a control ((**a**) *F. udum*, (**b**) *M. phaseolina*, (**c**) *R. solani*); (**d**, **e** and **f**) antagonistic effects of *Bacillus* isolates on *F. udum*; (**g**, **h** and **i**) antagonistic effects of *Bacillus* isolates on *M. phaseolina*; (**j**, **k** and **l**) antagonistic effects of *Bacillus* isolates on *R. solani*.

Rajgiri, Uttar Pradesh, Hariyana, Punjab belt and lastly West Bengal regions. One of the major factors for unequal distribution of biocontrol-based *Bacillus* may be the excess use of chemical pesticides in Punjab and Haryana region, as compared with other IGP regions, which causes disturbance in the soil physiochemical properties.[29] Scanning electron microscopic studies between pathogenic fungi and representative isolates (ZOI > 15 mm) by dual plate assay exhibited uneven growth of fungal mycelia as compared with control (healthy mycelia in absence of antagonists) (Figure 2).

Among these, strain TN10 has been found to inhibit the growth of all the three pathogenic fungi, while strains TN3 and NBIGP were effective against both *R solani* and *M. phaseolina*. Similarly strain TN8 was found to control *F. udum* and *M. phaseolina*, while strains NBIGP and NBIGP 24 have been found to produces inhibitory compounds causing damage to the fungal hyphae, as many researchers also reported *B. thuringiensis* produces sporulation parasporal crystals containing delta-endotoxins.[30–32] In our study, although the exact mode of action is not clear at present from scanning electron microscopy analysis, it may be concluded that antagonism may be offered by effect of secretion of antimicrobial or hydrolytic compounds from test organisms, which was justified by destruction caused by without any physical contact between antagonists and target pathogen.

Molecular characterization includes DNA fingerprinting (ARDRA analysis), and strain identification is important not only to design suitable biocontrol strategies but also for registration, patenting and recognition of biocontrol bacteria. ARDRA is a useful method for the identification, genotypic diversity and similarity of many prokaryotic microorganisms.[33–36] In our present study, molecular characterization based on 16S rDNA ARDRA analysis (Figure 3), with a set of three different restriction enzymes viz., *Alu*I, *Hae*III and *Msp*I with restriction sites AG′CT, GG′CC and C′CGG, respectively, revealed that a total of 140 biocontrol-based *Bacillus* isolates grouped into 26 representative clusters based on >50% similarity level of combined restriction pattern analysis (ARDRA analysis). Representative isolates from each cluster were selected for the identification by sequencing through Sanger's di-deoxy nucleotide sequencing method, and the sequences generated were analyzed by BLASTn followed by the closest

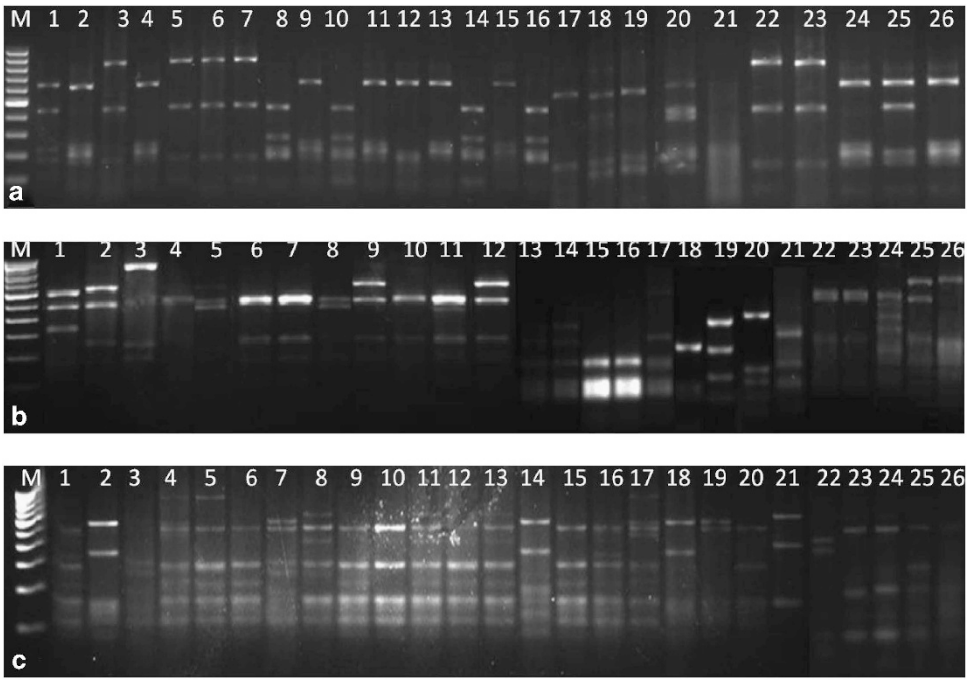

Figure 3 Representative RFLP gel showing PCR product of 16S rDNA digested with different restriction enzymes ((**a**) *Alu*I; (**b**) *Hae*III and (**c**) *Msp*I restriction endonuclease enzymes). Lane M contain the 100-bp DNA marker; lane 1, *B. licheniformis* strain NBIGPBL (accession no. JN004168); lane 2, *B. megaterium* strain NBIGP (accession no. JN004170); lane 3, *B. thuringiensis* strain NBIGP 24 (accession no. JN004169); lane 4, *B. fusiformis* strain TN1 (accession no. JQ415974); lane 5, *B. humi* strain TN2 (accession no. JQ415975); lane 6, *B. casamancensis* strain TN3 (accession no. JQ415976); lane 7, *Pontibacillus* sp. strain TN4 (accession no. JQ415977); lane 8, *Kurthia zopfii* strain TN5 (accession no. JQ415978); lane 9, *B. cereus* strain TN7 (accession no. JQ415980); lane 10, *B. arbutinivorans* strain TN8 (accession no. JQ415981); lane 11, *B. clausii* strain TN9 (accession no. JQ415996); lane 12, *Brevibacillus parabrevis* strain TN10 (accession no. JQ415982); 13, *Brevibacillus brevis* strain TN13 (accession no. JQ415985); lane 14, *B. subtilis* strain TN14 (accession no. JQ415986); lane 15, *B. drentensis* strain TN15 (accession no. JQ415987); lane 16, *Lysinibacillus sphaericus* strain TN16 (accession no. JQ415988); lane 17, *B. niacin* strain TN17 (accession no. JQ415989); lane 18, *B. fumarioli* strain TN18 (accession no. JQ415990); lane 19, *B. oleronius* strain TN19 (accession no. JQ415991); lane 20, *B. thermoamylovorans* strain TN20 (accession no. JQ415992); lane 21, *B. farraginis* strain TN6 (accession no. JQ415979); lane 22, *B. mycoides* strain TN12 (accession no. JQ415984); lane 23, *Paenibacillus polymyxa* strain TN21 (accession no. JQ415993); lane 24, *B. soli* strain TN22 (accession no. JQ415994); lane 25, *Paucisalibacillus globulus* strain TN23 (accession no. JQ415995); and lane 26, *B. koreensis* strain TN11 (accession no. JQ415983).

Table 2 Closest BLASTN matches for the full 16S rDNA sequences and their percentage similarity with the closest biocontrol-based *Bacillus* strains

S. No.	*Strain codes*	*Most similar type strain (%similarity/accession no.)*	*Most similar species (%similarity/accession no.)*	*Accession number*
1	NBIGP BL	*Bacillus aerius* strain 24K (99%/NR_042338.1)	*Bacillus licheniformis* strain ISA9 (99%/HQ189753.1)	JN004168
2	NBIGP	*Bacillus megaterium* strain IAM 13418 (99%/NR_043401.1)	*Bacillus megaterium* strain WN611 (99%/DQ275184.1)	JN004170
3	NBIGP 24	*Bacillus thuringiensis* strain IAM 12077 (99%/NR_043403.1)	*Bacillus thuringiensis* strain KVP109 (98%/JX290089.1)	JN004169
4	TN1	*Lysinibacillus boronitolerans* strain 10a (98%/NR_041276.1)	*Bacillus fusiformis* strain RSNPB4 (99%/HM588144.1)	JQ415974
5	TN2	*Bacillus humi* strain LMG 22167 (97%/NR_025626.1)	*Bacillus humi* strain NBIGP 23 (100%/JF304284.1)	JQ415975
6	TN3	*Bacillus shackletonii* strain LMG 18435 (97%/NR_025373.1)	*Bacillus casamancensis* (100%/AF519462.1)	JQ415976
7	TN4	*Pontibacillus marinus* strain BH030004 (95%/NR_043011.1)	*Pontibacillus* sp. strain BH85057-2 (100%/FJ897778.1)	JQ415977
8	TN5	*Bacillus fortis* strain R-6514 (95%/NR_042905.1)	*Kurthia zopfii* strain NBIGP 4 (99%/JF304286.1)	JQ415978
9	TN6	*Bacillus farraginis* strain R-6540 (99%/NR_025785.1)	*Bacillus farraginis* strain R-6915 (100%/AY443037.1)	JQ415979
10	TN7	*Bacillus thuringiensis* strain IAM 12077 (98%/NR_043403.1)	*Bacillus cereus* strain NBIGP 14 (99%/JF304293.1)	JQ415980
11	TN8	*Bacillus drentensis* strain IDA1967 (98%/NR_029002.1)	*Bacillus arbutinivorans* strain NBIGP 11 (100%/JF304294.1)	JQ415981
12	TN9	*Bacillus clausii* strain DSM8716 (99%/NR_026140.1)	*Bacillus clausii* strain NBIGP 3 (100%/JF304285.1)	JQ415996
13	TN10	*Brevibacillus parabrevis* strain IFO 12334 (97%/NR_040981.1)	*Brevibacillus parabrevis* strain NBIGP 2 (100%/JF304283.1)	JQ415982
14	TN11	*Bacillus korlensis* strain ZLC-26 (97%/NR_044538.1)	*Bacillus koreensis* (98%/FJ889614.1)	JQ415983
15	TN12	*Bacillus thuringiensis* strain IAM 12077 (97%/NR_043403.1)	*Bacillus mycoides* strain 820 (98%/FJ544336.1)	JQ415984
16	TN13	*Bacillus thuringiensis* strain IAM 12077 (99%/NR_043403.1)	*Brevibacillus brevis* strain NBIGP 7 (100%/JF304289.1)	JQ415985
17	TN14	*Bacillus subtilis subsp. subtilis* strain DSM10 (99%/NR_027552.1)	*Bacillus subtilis* strain ZM06 (99%/JF932296.1)	JQ415986
18	TN15	*Bacillus drentensis* strain IDA1967 (98%/NR_029002.1)	*Bacillus drentensis* strain YNB2 (99%/JN712311.1)	JQ415987

Table 2 (Continued)

S. No.	Strain codes	Most similar type strain (%similarity/accession no.)	Most similar species (%similarity/accession no.)	Accession number
19	TN16	*Lysinibacillus sphaericus* strain DSM28 (99%/NR_042073.1)	*Lysinibacillus sphaericus* strain VCRC B543 (99%/JN377786.1)	JQ415988
20	TN17	*Bacillus drentensis* strain IDA1967 (98%/NR_029002.1)	*Bacillus niacini* strain NBIGP 8 (100%/JF304290.1)	JQ415989
21	TN18	*Bacillus fumarioli* strain LMG17489 (99%/NR_025370.1)	*Bacillus fumarioli* strain R-14705 (99%/AJ581126.1)	JQ415990
22	TN19	*Bacillus oleronius* strain ATCC 700005 (99%/NR_043325.1)	*Bacillus oleronius* strain 11 (99%/EU430987.1)	JQ415991
23	TN20	*Bacillus thermoamylovorans* strain DKP (99%/NR_029151.1)	*Bacillus thermoamylovorans* strain BHK180-4 (99%/AB360824.1)	JQ415992
24	TN21	*Paenibacillus jamilae* strain CECT 5266 (98%/NR_042009.1)	*Paenibacillus polymyxa* strain M1 (99%/HE577054.1)	JQ415993
25	TN22	*Bacillus soli* strain R-16300 (99%/NR_025591.1)	*Bacillus soli* strain LMG 21839 (99%/AJ542514.1)	JQ415994
26	TN23	*Ornithinibacillus californiensis* strain MB-9 (98%/NR_041820.1)	*Paucisalibacillus globulus* strain 5 (100%/EU430986.1)	JQ415995

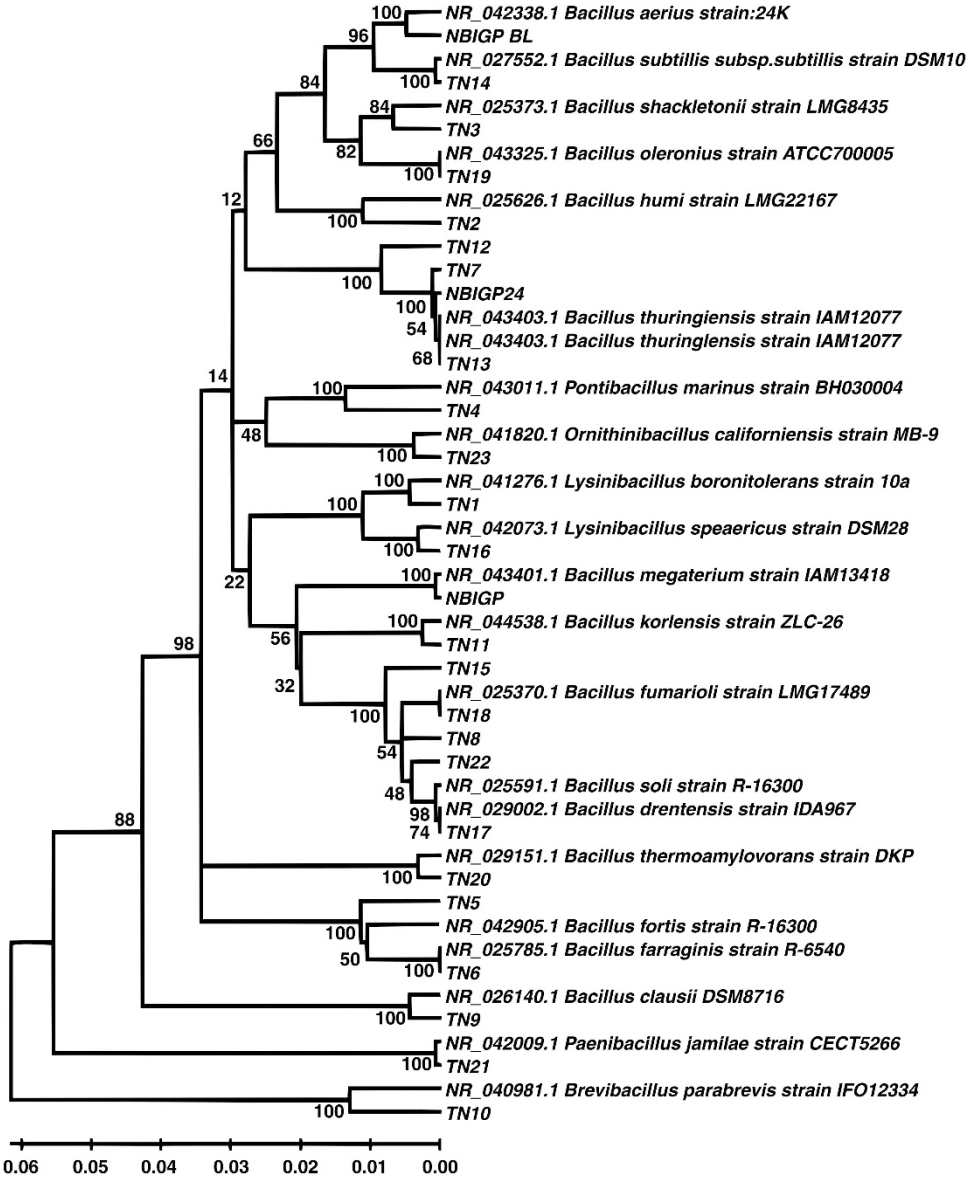

Figure 4 Phylogenetic tree of 16S rRNA sequences from selected isolates by unweighted pair group method of arithmetic mean method. The sequence data for several closely related *Bacillus* isolates were recovered from genebank and included in the tree. The accession numbers and their sequences recovered from genbank are as follows: the boot strap values from 5000 pseudoreplications are shown at each of the branch points on the tree. Bar indicates % similarity.

taxonomic affiliation of the sequences matched from public database (NCBI, Genbank) (Table 2), and results revealed that strains TN4, TN5, TN2, TN11 and TN3 are distantly related to type strains of previous known species with 95, 95, 97, 97, 97% of similarity, respectively. Further phylogenetic analysis was carried out by construction of dendogram of all the representative isolates and compared with the submitted 16S rRNA sequences in public database (Figure 4). This phylogenetic tree revealed that the biocontrol potential *Bacillus* isolates were phylogenetically diverse in the genus *Bacillus* and related genera, which belonged to the two families.

In conclusion, the results showed an evidence of high diversity of *Bacillus* strains in rice–wheat rhizosphere ecosystem at IGP. A total of 26 representative *Bacillus* isolates showed antagonism against *M. phaseolina*, *F. udum* and *R. solani* and were phylogenetic diverse in nature. The diversity analysis of *Bacillus* with respect to antagonistic behaviour had enriched our knowledge in most fertile regions of India. Our study further increases the information of *Bacillus* species showing biocontrol activity (evident by scanning electron microscopy), which was available for developing suitable biocontrol strategies as well as enable us for the recognition of biocontrol bacteria from rhizospheric soil of rice–wheat ecosystem.

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