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Identification and molecular phylogeny analysis using random amplification of polymorphic DNA (RAPD) and 16SrRNA sequencing of N_2 fixing tea field soil bacteria from North Bengal tea gardens

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Random amplification of polymorphic DNA (RAPD) amplification genomic DNA of 23 selected laboratory cultures of bacteria using RAPD revealed their polymorphism. Polymerase chain reaction (PCR) amplification of the bacterial 16SrDNA was performed using 704F GTAGCGGTGAAATGCGTAGA and 907R CCGTCAATTCCTTTGAGTTT primer, sequenced and accessed in NCBI (No. KY636356, KY631488, KY 860028, KX587470, KX665547, KY631489, KX608591, KY636360, KY671245, KY631490, KX587469, KY859856, KX665546, KX608590, KX587468, KY859798, KY636357, KY636361, KY 636359, KY631491, KY 859855, KY636358, KY636362) after submitting the contig. FASTA sequence in NCBI database was seen. All most all 23 bacterial strains (viz. TS-1-16, TS-4-23 DJ-1-22, DS-1-20, AS-1-4, DJ-1-24 , DJ-1-10, DJ-1-46) showed strong homology with free living nitrogen fixing soil bacteria, also showing (98 to 100%) identity and E-value of 00 with *Burkholderia* spp, Strain-S-9-19, Str-S-9-15, *SP-2386*, *Stenotrophomonas maltophilla, strn-MM-3-3, Str-D-3,LP-05*, *Bacillus cereus Strn*-FORC021and *Azospirillum* sp *TSH51* gene, having good nitrogen fixing capacity. Phylogenetic tree analysis among the 23 isolates and between the different strains from GenBank showed close similarity. Most of the isolated bacterial strain identified as a member of the genus *Burkholderia* sp, *Stenotrophomonas maltophila, Herbaspirillum* sp, *Acinetobacter johnsonii, Methylobacter, SP-T-20*, *and Bacillus cereus, Azospirillum* sp would be consider to be the most suitable bioferiliser for organic and conventional tea gardens of North Bengal, India.

Key words: Molecular phylogeny, RAPD, 16SrRNA sequencing, free living, N_2 fixing soil bacteria.

Introduction

Tea *Camellia sinensis* (L) O. "Kuntze" of family *Theaceae* is the most commonly used beverage in India and in the world. Tea is an evergreen shrub that mainly grows in tropical and subtropical areas. It is thought to have

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originated in East Asia somewhere between China and Burma. India is the world's second largest tea producer country next to China. In the financial year 2015 to 2016, India has recorded tea production of 1,233 million kg (mn kg) and exports crossing 230 mn kg after 35 years. The top five teas producing countries are China, India, Kenya, Sri Lanka and Turkey (http://www.gktoday.in/blog/key-facts-about-tea-production-in-india/). India has around 563.98 thousand hectares of tea cultivated land (December 2013). Assam is the highest Indian agricultural soil that contains low nitrogen and cellulose, and therefore the self-sustaining free-living nitrogen-fixing micro flora would be of great advantage if their identity is known and their ability is properly exploited. The reduction of chemical fertilizers by the application of biological fertilizers is mainly based on the bacteria involved in nitrogen fixation as one of the suitable steps in sustainable agriculture (Vejan et al., 2016).

The plant growth promoting rhizobacteria (PGPR) microorganisms play beneficial effects on the plant health (Philippot et al., 2013) directly by nitrogen fixation, different phytohormone production, phosphate solubilisation and iron sequestration by siderophore production and indirectly by plant growth stimulation by producing antifungal metabolites preventing different phytopathogens (Glick and Bashan, 1997). Diverse bacterial genera such as *Arthrobacter, Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Klebsiella*, *Burkholderia*, *Erwinia*, *Flavobacterium*, *Micrococcous*, *Enterobacter*, *Xanthomonas*, *Chromobacterium*, *Serratia* and *Caulobacter* spp have been reported to increase plant growth (Bhattacharyya and Jha, 2012; Bal *et* al., 2013). Rhizospheric *Azospirillum* sp of many grasses and cereals is a well known PGPR all over the world. Presently, N_2 fixing PGPR that have plant stimulation includes the endophytes that is, *Azoarcus*, *Burkholderia*, *Herbaspirillum* spp and *Gluconacetobacter diazotrophicus*, and the rhizospheric bacteria *Azotobacter*sp and *Paenibacillus (Bacillus) polymyxa* (Vessey, 2003).

In recent times, PGPR got more attention and it has been used as potent biofertilizers (Richardson et al., 2009; Compant et al., 2010) as prolonged use of chemical fertilizers is perilous to soil, as well as, human health and also detiorate the crop quality (Islam et al., 2013). Alternative biotechnological approaches are adapted in different agriculture practices to not only increase the crop production and plant growth, but also to maintain soil health. It has been reported that inoculation of *Azospirillum* biofertiliser or liquid near the rhizosphere of tea significantly increased growth. Although, research about PGPR impact on the tea plants is still poorly organized, especially in the Northeast region of India including North Bengal tea growing region. The productivity of tea is decreased remarkably due to intensive application of chemical fertilizers for a prolonged period (Sharma et al., 2014).

Therefore, there is a growing demand to explore the indigenous micro flora associated with the tea rhizosphere soil not only to reduce the application of chemical fertilizer, but also for the benefit of plant, soil health and the environment. The 16S rRNA represents the right candidate to study bacterial evolution, ecology, phylogenetic relationships among taxa, bacterial diversity and quantification of the relative abundance of taxa of various ranks (Hugenholtz et al., 1998). Whereas, random amplified polymorphic DNA (RAPD) fingerprinting explores genetic polymorphisms (Teaumroong and Boonkerd, 1998) in bacteria. RAPD fingerprinting has been used for strain identification and to determine the genetic diversity within a field population of pink-pigmented facultative methylotrophs (Balachandar et al., 2008), *Rhizobium* isolates (Rajsundari et al., 2009), *Photorabdus* and *Xenorabdus* isolates (Moghaieb et al., 2017).

There was scanty report on molecular identification of free living N_2 fixing PGPR of North Bengal tea gardens of West Bengal. A preliminary investigation on isolation and characterization of free-living soil bacteria from tea gardens of Terai, Dooars and Darjeeling district West Bengal have been carried by the present research group. Morphological and biochemical evaluation of free living N_2 fixing tea rhizospheric and tea soil bacteria of North Bengal tea gardens has also been investigated (Bhaduri et al., 2018).

Keeping the background information, the present study has been undertaken for molecular identification and to understand the genetic diversity of free living N_2 fixing soil bacteria from tea garden soil of North Bengal to be used as biofertiliser.

MATERIALS AND METHODS

Pure cultures of previous study (Bhaduri et al., 2018) were used as experimental sample for this investigation, 23 strains for 16SrRNA analysis and 22 strains for RAPD analysis (El-Fiki, 2006).

Isolation of genomic DNA

Genomic DNA was isolated from selected pure bacterial isolates of three different region sample screened on the basis of salt tolerance, antibiotic resistance total N content, etc. following the cetyl trimethylammonium bromide (CTAB) method (Gomes et al., 2000). The isolated genomic DNA was treated with RNase and then subjected to Agarose Gel (0.8%) electrophoresis to check the purity of DNA.

RAPD

Random Amplification of Polymorphic DNA of selected strains to observe the genetic variability between them was carried out at Xcelris Lab. Ahmedabad, Gujrat using two RAPD primer P1v(5' to 3'): GTG TGT GTG TGT GTG TGT GT, (20) nts., El-Fiki 2006 and P2:OPQ1 (5' to 3'): GGGACGATGG (10) nts (Balachandar et al.,

2008; Rajsundari et al., 2009; Moghaieb et al., 2017). PCR was carried out in a final reaction volume of 25 µl in ABI Veriti Thermal Cycler. Amplification reactions were performed in a 25 µL volume, containing: 20 mM Tris-HCl (pH 8.4), 50 mMKCl, 2.5 mM $MgCl_2$, 200 µM each of dNTPs, 1 µM primer, 30 ng of genomic DNA and 1.5 U of *Taq* DNA polymerase. The reaction mixture was flooded with two drops of mineral oil, initial denaturation for 5 min at 95°C, the amplification, then continued for 35 cycles consisting of 30 s at 94°C, 30 s at 36°C and 60 s at 72°C followed by a 7 min final extension at 72°C. Amplification product was separated by gel electrophoresis on precast 1.2% agarose gel and visualized under ultra-violent (UV) illumination after staining with ethidium bromide and Gel Documented on Gel Documentation System (Lee et al., 2012).

PCR amplification of 16SrRNA gene

PCR amplification of 16SrRNA was performed using 8F AGAGTTTGATCCTGGCTCAG. 1492R ACGGCTACCTTGTTACGACTT and sequencing of isolated bacterial 16SrRNA was performed using 704F GTAGCGGTGAAATGCGTAGA and 907R CCGTCAATTCCTTTGAGTTT primer. PCR amplification conditions: DNA1 µl, 16S Forward Primer 400ng, 16S Reverse Primer 400ng, dNTPs (2.5 mM each) 4 µl, 10X Taq DNA polymerase Assay Buffer 10 µl,Taq DNA Polymerase Enzyme (3U/ µl) 1 µl,Water X µl,Total reaction volume 100 µl. The PCR was conducted at 95°C, 94°C, 50°C ,72°C, 72°C, 5 min, 30 s, 30 s, 1 min 30 s and 7 min respectively for 35 cycles (protocol followed by Xcelris Lab) .

Sequencing of 16S rRNA

The PCR amplicon (1.4 kb approximately) was purified with ExoSap enzymatic purification as per the manufacturer's instruction (ABI). After the purification, the products were subjected to Sanger sequencing using ABI, 3730XL DNA analyzer using BdT v3. 1 chemistry. Each forward and reverse reaction of PCR amplified products were sequenced separately. Forward and Reverse DNA sequencing reaction of PCR amplicons of respective samples was carried out using BDT v3. 1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Construction of phylogenetic tree

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura et al. (2004) model. Evolutionary genetics analysis uses maximum likelihood, evolutionary distance, and maximum parsimony methods were conducted in MEGA 5 software (Tamura et al., 2011). The RAPD profile derived phylogeny was performed by Xcelris Lab.

RESULTS AND DISCUSSION

Purified genomic DNA isolated from bacterial strains after resolving in 0.8% agarose gel reveals their good yield and large genome size (Figure not shown).

PCR amplification of 16SrRNA gene and sequencing

Twenty three isolated bacterial genomic DNA was amplified with forward and reverse sequencing primer. PCR amplified fragments of approximately 1.4 kb in size are sequenced.

GenBank accession followed by homology searching

Twenty three (23) GenBank Accession were obtained after submitting the contig FASTA sequence. Most of the 23 isolated bacterial strain demonstrated strong homology with known nitrogen fixing bacteria. The strain TS-1-16 (Accession No. KY636356) displayed 100% homology with *Burkholderia* sp, Strain-S-9-19 (Accession No. KY357337) having nitrogen fixing capacity was ascribed as a member of the genus *Burkholderia*, since our sequence was not full length. TS-4-23 (Accession No. KY631489) showed 100% identity along with *Burkholderia* sp, Str-S-9-15 (Accession No. KX212131) having good nitrogen fixing capacity**.** DS-1-20 (Accession No. KY636360) showed 100% identity with *Stenotrophomonas maltophilla, strn-MM**-3-3,*** (Accession No. KT970988) having nitrogen fixing capacity and the other *Stenotrophomonas* species, hence it is ascribed as a member of the genus *Stenotrophomonas*. DJ-1-22 (Accession No. KY 859855) showed 100% identity with *Burkholderia SP-2386*, (Accession No.JX174263) having good nitrogen fixing capacity. AS-4 (Accession No.KY636361) showed 100% identity with *S. maltophilla, Str-D-3* (Accession No. KM488439) having nitrogen fixing capacity. The bacterial strains DJ-1-24 (Accession No. KY636358) showed 100% identity with *S. maltophilla-LP-05* (Accession No. KT427904), DJ-1-10 (Accession No. KY631491) 99% identity with *Bacillus cereus Strn*-FORC021 (Accession No. CP-014486), DJ-1-46 (Accession No. KY636362) 98% identity and with *Azospirillum*sp *TSH51 gene*, (Accession No. AB508854) having nitrogen fixing capacity, the latter is well known free living nitrogen fixing soil bacteria (Table 1).

Evolutionary genetics analysis and phylogenetic tree

Phylogenetic tree analysis between the 23 isolated strains showed close similarity among the strains. The tree reveals that there are 8 main groups consisting of two closely related strains. The group 1 consists of strains of TS-4-23 and DS-1-16 which resembles the *Burkholderia* sp functioning as a free living N_2 fixer and belonging to PGPR activity (Hayat et al., 2010).

Grou p 2 contained strains of TS-4-12 and DJ-1-46 which resembles the *Burkholderia cepacia* and *Azospirillum* sp *TSH51* gene, which solely functions as a free living N_2 fixer and displaying PGPR activity. Group 3 contained strains of DJ-1-3 and DJ-1-22 which resembles the *Burkholderia* sp different strain which solely functions as a free living N_2 fixer and PGPR activity. Group 4 contained strains of AS-4 and DJ-1-24 which resembles

Table 1. Homology and annotation of 23 GenBank accession of isolated N_2 fixing Bacterial strain.

Strain code	NCBI accession No	Homology	Length (bp)	Annotation	Max score	Total score	E-value	Identical (%)
TS-1-16	KY636356	KY357337	1439	*Burkholderia*sp, Strain-S-9 19,Nitrogen fixation	2658	2658	00	100
TS-3-15	KY631488	KX3500422	1202	*Stenotrophomonasmaltophila* ,Str-F-4-2-35,Nitrogen fixation.	2220	2220	00	100
TS-4-12	KY 860028	KX055886	1309	*Burkholderiacepacia*str-TY-5-SH,Nitrogen fixation	2418	2418	00	100
TS-3-27	KX587470	KY266795	934	Bacterium str-CH-2,Nitrogen fixation.	1447	1447	00	97
TS-4-16	KX665547	KP276241	611	*Herbaspirillumsp*str hz-10,Nitrogen fixiation	2658	2658	16-61	78
TS-4-23	KY631489	KX212131	1186	*Burkholderia sp,*Str-S-9-15 ,Nitrogen fixation	2191	2191	00	100
TS-4-24	KX608591	DQ257427	620	*Acinetobacter johnsonii*, CA-1-14-,Nitrogen fixation	697	697	00	88
DS-1-20	KY636360	KT970988	1473	*Stenotrophomonasmaltophilla, strn-MM-3-3*,Nitrogen fixation	2721	2721	00	100
DS-1-16	KY671245	KF826288	1451	*Burkholderiacenocepacia* , Nitrogen fixation	2680	2680	00	100
DS-1-18	KY631490	KP216607	1412	*Burkholderiaanthina*strn-JT N301, Nitrogen fixation	2189	2189	00	98
DS-1-25	KX587469	KY810619	928	*Burkholderiacenocepcia*, StrKNL-15, Nitrogen fixation	1504	1504	00	97
DS-1-26	KY859856	CP-007747	1105	*Burkholderiacepacia* ,ATCC-25416Nitrogen fixination	1882	1882	00	98
DS-2-8	KX665546	AF131868	552	*Methylobacter,SP-T-20*, Nitrogen fixation	324	324	2e-84	78
DS-2-9	KX608590	EU 705721	742	*Uncultured Ralstonia*sp*, Clone-3P-3-2*, Nitrogen fixation	477	477	2e-130	79
DS-2-10	KX587468	NR114140	918	*HerbaspirillumfrisingenseStr -NBRC 102-522,* Nitrogen fixation.	538	538	1e-148	81
AS-1	KY859798	KT580654	1200	*Stenotrophomonasmaltophilla ,strn*-Can R-47,Nitrogen fixation	2109	2109	00	98
AS-2	KY636357	HM246521	1434	*Stenotrophomonasmaltophilla , strn-6*,Nitrogen fixation	2644	2644	00	100
AS-1-4	KY636361	KM488439	1412	*Stenotrophomonasmaltophilla, Str-D-3*,Nitrogen fixation	2577	2577	00	99
DJ-1-3	KY 636359	KY357336	1432	*Burkholderia Strn-S-9-18*, Nitrogen fixation	2645	2645	00	100
DJ-1-10	KY631491	CP-014486	1531	*Bacillus cereus Strn* FORC021,Nitrogen fixation	2693	2693	00	99
DJ-1-22	KY 859855	JX174263	1428	*Burkholderia SP-2386*, Nitrogen fixation	2638	2638	00	100
DJ-1-24	KY636358	KT427904	1412	*Stenotrophomonas maltophilla-LP-05*, Nitrogen fixation	2593	2593	00	100
DJ-1-46	KY636362	AB508854	1416	*Azospirillumsp TSH51 gene*, Nitrogen fixation	2420	2420	00	98

the *S. maltophilla* different strain which solely functions as a free living N_2 fixer and PGPR activity (Fouzia et al., 2015).

Group 5 contained strains of TS-3-15 and DS-1-20 which resembles the *S. maltophilla* different strain which solely functions as a free living N_2 fixer and PGPR activity. Group 6 contained strains of TS-3-27 and DJ-1-10 which resembles the Bacterium str-CH-2 and *Bacillus cereus strn*-FORC021which solely functions as a free living N_2 fixer and PGPR activity. Group 7 contained strains of TS-4-16 and DS-2-10 which resembles *Herbaspirillum* sp different strain which solely functions as free living as well as endophytic (certain strain) N_2 fixer and PGPR activity in tea plant (Zhan et al., 2016).

Group 8 contained strains of DS-2-8 and DS-2-9 which resembles *Methylobacter, SP-T-20* and uncultured *Ralstonia* sp., *Clone-3P-3-2* which solely functions as a free living N_2 fixer and PGPR activity. The other seven strains are distantly related to these clusters having nitrogen fixing and plant growth promoting activity (Figure 1) (Hayat et al., 2010).

RAPD

RAPD analysis of isolated bacterial genomic DNA reveals a little polymorphism pattern (Figure 2). Among the two primers tested only primer P1 was proper for amplification. The bacterial isolates DS-2-10; DS-2-8; DS-1-25; TS-4-24 gave no response

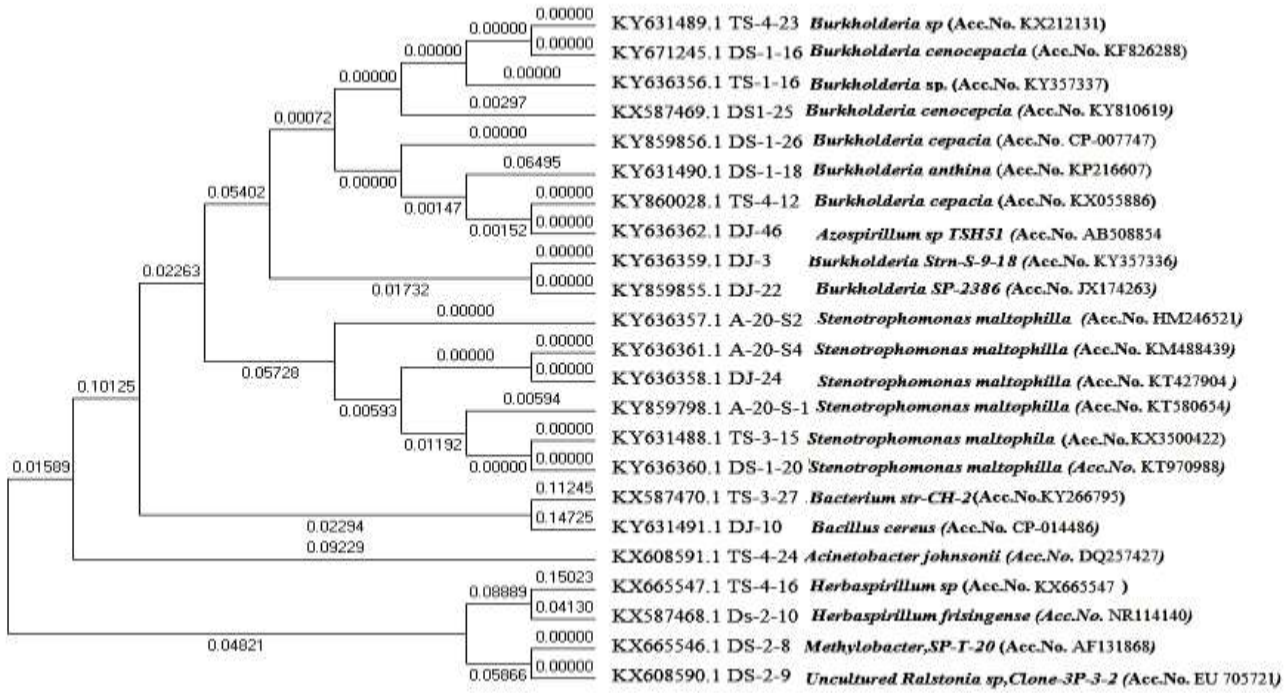

Figure 1. Phylogenetic tree between the 23 isolated bacterial strain showing homology (interrelationship) between them and highest matching with the GenBank Accession in National Center for Biotechnology Information.

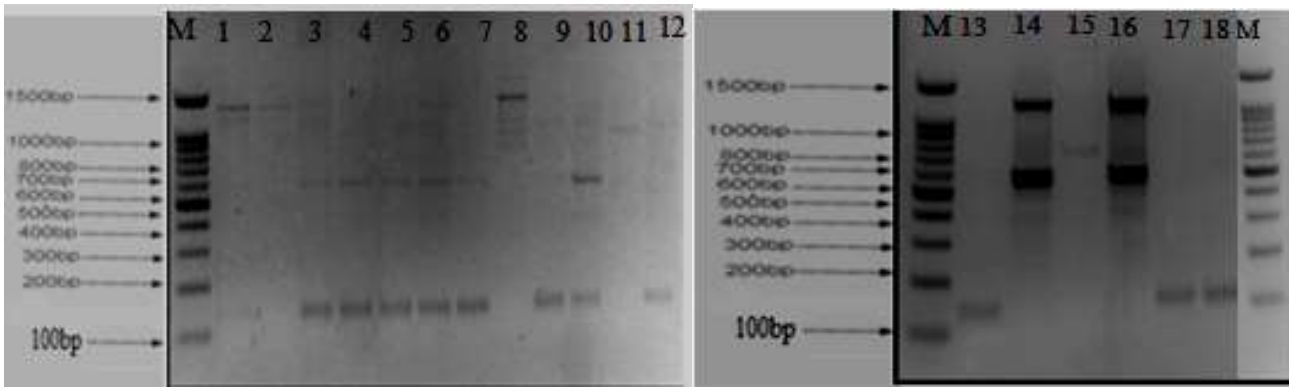

Figure 2. Representative RAPD profile with primer P1, M. 100 bp DNA Ladder of 1.5 kb size; 1. TS-3-15; 2. DJ-1-10; 3. DS-1-20; 4. DS-1-26; 5. DS-1-16; 6. TS-4-12; 7. DS-1-18; 8. DJ-1-22; 9. TS-1-6; 10. TS-4-23; 11. DJ-1-3; 12. AS-1-4; 13. DJ-1-46; 14. DJ-1-24; 15. AS-1; 16. AS-2; 17. DS-2-9; 18. TS-3-27.

at RAPD amplification and rest of the 18 isolates showed amplification. The DNA amplified fragment varied in size ranges from 100 bp to 1.5 kb. The dendrogram result of polymorphic band showed similarity between the organisms as exhibited by 18 strains (Figure 3). The strain DS-1-20 is closely related to the cluster of DS-1-18, TS-4-12, DS-1-16 and DS-1-26. The strain AS-1-4 shows similarity to cluster containing TS-1-6 and TS-4-23, the former is closely related to the cluster formed by TS-3-27, DJ-1-46 and DS-2-9. The strains DJ-1-10 and TS-3-15 are closely related to each other and distantly related to a cluster formed by AS-1-2 and DJ-1-24, the latter two are related to a cluster formed by DJ-1-22 and DJ-1-3. AS-1-1 strain could not produce polymorphism and hence cannot relate to the cluster (Figure 3).

The genus *Burkholderia* comprises of 19 species, which includes soil and rhizosphere bacteria as well as plant and human pathogens (Bevivino et al., 1998; Achouak et al., 1999; Zhang et al., 2000; Balandreau et al., 2001). The aerobic, rod-shaped, endospore producing

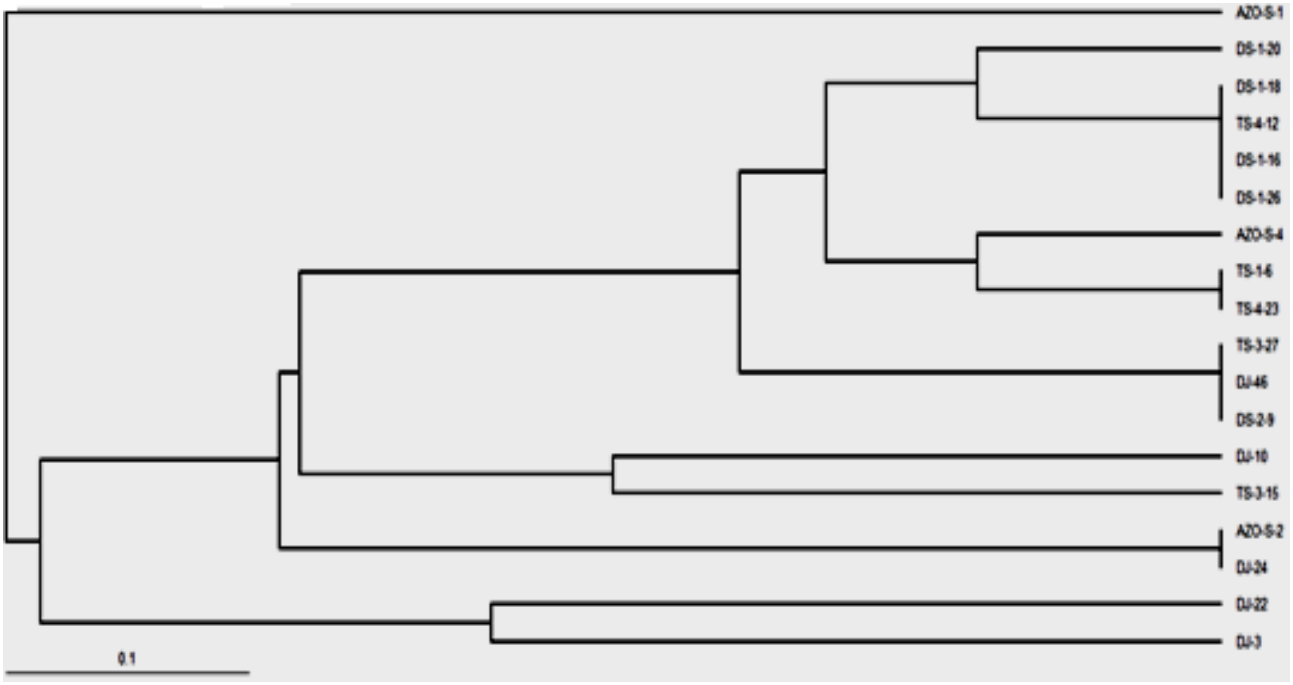

Figure 3. Total homology analysis of 22 Bacterial Genomic DNA derived from RAPD profile showing their genetic relatedness.

genus *Bacillus* is a systematically diverse taxon (Claus and Berkeley, 1986). Gene sequence analyses (16SrRNA) have identified at least 10 phylogenetic groups in the genus Bacillus (Shida et al., 1997a). *Bacillus cereus* AR156 having PGPR activities induces systemic resistance in *A. thaliana* by simultaneously activating salicylate- and jasmonate/ ethylene-dependent signaling pathways which has been established (Niu et al., 2011). *B. cepacia* is recognized for its abilities, to promote maize growth (Bevivino et al., 1998), to enhance crop yields (Chiarini et al., 1998), and to suppress many soilborne plant pathogens (Bevivino et al., 1998; Hebbar et al., 1998; McLoughlin et al., 1992), as well as to degrade diverse pesticides (Daubaras et al., 1996; Mueller et al., 1997).

The genus *Stenotrophomonas* comprises of about eight species. Strains of the most common species, *Stenotrophomonas maltophilia*, have a function that includes beneficial effects of plant growth (Ryan et al., 2009). *S. maltophilia* is an ubiquitous, aerobic, non-fermentative and Gram-negative bacillus that is closely related to the *Pseudomonas* species (Calza et al., 2003). The genus *Stenotrophomonas* has pathogenic effect and they are resistant to certain antibiotics and susceptible to Chlorampenicol which we have already investigated in our previous study. Nahi et al. (2016) studied the effect of herbicide on nitrogenase and N_2 fixing capacity of *Stenotrophomonas maltophilia* (Sb 16). Bacteria of the genus *Azospirillum* (α-subclass of Proteobacteria) are known as plant growth promoting rhizobacteria (Okon, 1994. They were isolated from the rhizosphere of many grasses and cereals all over the world, in tropical as well as in temperate climates (Patriquin et al., 1983). Due to cell shape, growth behavior and habitat within grass roots, genus *Herbaspirillum* were previously thought to be a new *Azospirillum* species. However, RNA-RNA hybridization experiments reveal no relationship with *Azospirillum* spp or *Aquaspirillum itersonii* (Falk et al., 1986). *Herbaspirillum seropedicae*, *Herbaspirillum frisingense* and *Herbaspirillum lusitanum*able are reported to fix nitrogen (Baldani et al., 1986; Kirchhof et al., 2001; Valverde et al., 2003). The endophytic *Herbaspirillum* sp WT00C isolated from the tea plant, seems to have a potential ability to promote tea-plant rooting and budding due to its capability of producing indole-3-acetic acid (IAA), ammonia and siderophores (Zhan et al., 2016). Bacterial species of the genus *Acinetobacter* are ubiquitous in nature (Bergogne-Berezin and Towner, 1996).

In recent years, members of the genus *Acinetobacter* have been isolated from the rhizo- sphere of different plants (Kuklinsky-Sobral et al., 2004; Roberts et al., 2005; Nakayama et al., 2007; Li et al., 2008). In India, *A. indicus* was described for the first time in soil samples collected from hexachlorocyclohexane dump sites (Malhotra et al., 2012). Strains belonging to the genus *Acinetobacter,* and their plant growth-promoting properties have been reported in the literature (Sachdeva et al., 2010). The presence of different species of *Acinetobacter* was worked in the rhizosphere of three agricultural wheat fields of Pune, India. The genetic diversity of *Acinetobacter* species using metagenomics

study in the wheat rhizosphere was assessed by denaturing gradient gel electrophoresis (DGGE) of 16 SrRNA genes PCR products. Plant growth-promoting traits such as nitrogen fixation, siderophore production and mineral solubilization were reported in *in vitro* culture of *Acinetobacter* isolates (Sachdeva et al., 2010). From the perusal of literature it has been revealed that, in India no work has been done with *Acinetobacter* sp in tea field soil for their study related to biofertiliser or PGPR.

Auman et al. (2001) reported *nitrogenous* and utilize N_2 as a nitrogen source by some methane-oxidizing bacteria (methanotrophs). There are two types of methanotrophs - type I and type II. Type II methanotrophs and members of the type I genus *Methylococcus*have been shown to be capable of nitrogen fixation, while type I methanotrophs are not (Dedysh et al., 2000; Murrell and Dalton, 1983; Oakley and Murell, 1988). The genus *Ralstonia* established in 1995 by Yabuuchi et al. (1995) accommodate species previously known as *Alcaligeneseutrophus*, *Pseudomonas solanacearum* and *Pseudomonas pickettii*. *Ralstonia eutropha*isolated from sludge, soil and *R*. *basilensis* from waste-water (Steinle et al., 1998). Chen et al. (2001) isolated several strains of *Ralstonia* from *Mimosa* as a symbiont nitrogen fixer, the most promising one is *Ralstonia taiwanensis*, cells are Gram negative, non spore forming rod shaped and mean cell size which ranges from 0.5 to 0.7 m width and 0.8 to 2.0 m in length (Chen et al., 2001).

Gulati et al. (2011) reported the presence of Gram-negative nitrogen fixing bacteria of α-Proteobacteria genera *Brevundimonas*, *Rhizobium*, and *Mesorhizobium*; γ-Proteobacteria genera *Pseudomonas* and *Stenotrophomonas*; and β-Proteobacteria genera *Azospira*, *Burkholderia*, *Delftia, Herbaspirillum and Ralstonia* associated with the tea roots of Kangra Valley of Himachal Pradesh. The isolated bacterial strain identified as *Burkholderia*sp, *Stenotrophomonas maltophila, Herbaspirillum* sp, *Acinetobacter johnsonii, Methylobacter, SP-T-20*, *and Bacillus cereus, Azospirillum* sp depicts the N_2 fixing as well as PGPR activities as evident from homology searching can be used as potent bioferiliser for organic and conventional tea gardens especially in North Bengal. The present investigation will suggest an insight to the tea growers of North Bengal and researchers as readily established “Bio accelerant” or” Bio fertiliser”.

Conclusion

Since tea is a non-leguminous plant, the search for free living N_2 fixing soil bacteria in tea growing areas is gaining momentum day by day. Few good strains have been identified to be used as a potential N_2 fixer in tea field. The isolated bacterial strain identified as a member of the genus *Burkholderia* sp, *Stenotrophomonas maltophila, Herbaspirillum* sp, *Acinetobacter johnsonii, Methylobacter, SP-T-20*, *and Bacillus cereus, Azospirillum* sp can be the right candidates as potent bioferiliser for organic and conventional tea gardens of North Bengal, India. Hence, it is evident from the homology searching that our isolated strain would be ascribed as a member of the respective genus until and unless DNA-DNA hybridization and other biochemical parameter have been tested. The study reveals a thorough investigation regarding the molecular identification of free-living N_2 fixing bacteria; however, their field application is still needed in further study.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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