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Research Article

Identification and Biochemical Characterization of β-Rhizobia isolated from Root Nodules of *Mimosa pudica*

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Abstract

Total ten Gram negative, single short rod shaped and motile bacterial strains were isolated from the roots of *Mimosa pudica* from different regions of Sukna forest in North Bengal, India. Isolates were characterized based on biochemical and 16S rRNA sequence analysis and it was found that all had close relationship to members of the genus *Paraburkholderia*, named *Paraburkholderia caribensis* (MWAP64T). Strains grew well in the yeast extract mannitol agar (YEMA) medium (pH 7.0) at 30°C and showed negative results to catalase, ketolactase and cellulose test. Plant growth promoting abilities of the bacterial isolates were identified by conducting various tests. After pot nodulation experiment, six isolates were considered as true *Paraburkholderia* (SK ND1, SK ND2, SK ND3, SK ND4, SK ND8 and SK ND9). Optimum growth temperature was observed at 20°-40°C, at pH 5.0-8.0 and NaCl concentration of 1-3% (W/V).

Keywords: *Mimosa pudica,* root nodules, Sukna forest, *Paraburkholderia caribensis*, pot nodulation.

Introduction

Plant require nitrogen in large quantities for synthesizing amino acids, proteins, nucleic acids and other nitrogenous components essential for their growth and development. Plants cannot use atmospheric nitrogen directly until it is converted in to ammonia (NH_4^+) or nitrate (NO_3^-). Biological Nitrogen Fixation (BNF) is a process in which atmospheric nitrogen is converted into form which is directly assimilated by plants. Rhizobia are the common name used for diazotrophic symbiotic bacteria and its 16 genera belong to the class α-proteobacteria, while *Paraburkholderia, Trinickia* and *Cupriavidus* belong to the β-proteobacteria (Estrada *et al*., 2018). Earlier, *Paraburkholderia* was classified as *Burkholderia* and belong to the Burkholderiaceae family, in the order Burkholderiales. The genus *Burkholderia* was first reported by Yabuuchi *et al*. (1992). Pandey *et al.* (2005) in Dehradun and Chen *et al*. (2001) in Taiwan reported the presence of β-rhizobia in the root nodules of *Mimosa pudica.* The genus *Burkholderia* invite attention of the many scientists worldwide because of its some outstanding properties, including a high level of diversity, possessing great metabolic diversity and with many strains showing biotechnological potential (Eberl and Vandamme, 2016). According to Burkholderia sensu lato, the genus reclassified (based on the phylogenetic analysis of the 16S rRNA and housekeeping genes) into two major clades, one is clinically important and phytopathogenic member and the other one is environmental beneficiary species (Gyaneshwar *et al*., 2011; Estrada *et al*., 2013). A detailed phylogenomic study of 45 *Burkholderia* species resulted in the proposal of two new genus, *Paraburkholderia* and *Caballeronia* (Dobritsa and Samadpour, 2016). These two genera were subdivided into four more genera, including *Trinickia*, *Caballeronia*, *Robbsia* and *Mycetohabitans* (Estrada *et al*., 2018). In this reclassification, five *Paraburkholderia* strains were shifted to the genus *Cabellaronia* (*P. glathei, P. grimmiae*, *P. humi*, *P. sordidicola and P. zhejiangensis*), three to *Trinickia* (*P. caryophylii, P. soli and P. symbiotica*), two to *Mycetohabitans* (*P. endofungorum and P. rhizoxinicia*) and one to *Robbsia* (*P. andropogonis*) (Estrada *et al*., 2013; Dobritsa and Samadpour, 2016).

Currently, *Paraburkholderia* is composed of 74 recognized species from the most various environments, including agricultural soils, water, and root nodules of plants (Farh *et al*., 2015; Weber and King, 2017). Some *Paraburkholderia* species have been isolated from root nodules of plants of the Mimosoideae family, especially of the genus Mimosa (Sheu *et al*., 2013; Paulitsch *et al*., 2019). Mimosa is the second largest genus of the subfamily Mimosoideae containing 540 species, mostly distributed in neotropical regions (Barneby, 1991).

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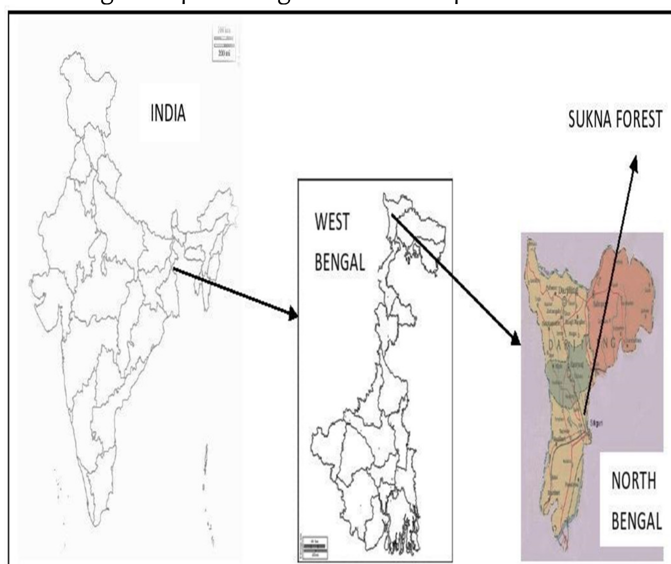
Fig. 1. Map showing the site of sample collection.



According to recent classification, mimosas would fit into the subfamily Caesalpinioideae, in the clade Mimosoideae–Caesalpinieae–Cassieae (MCC) (LPWG, 2017). Most studies of mimosoid symbionts reported in Brazil and found that *Paraburkholderia* are the preferred symbionts (Junior *et al.*, 2010; Paulitsch *et al.*, 2019). *Paraburkholderia* genus represents diversity as it has ability to nodulate many species of Mimosa, Calliandra, and Piptadeniae plants (Bournaud *et al.*, 2013; Sheu et al., 2013; Moulin *et al.*, 2015; Dall'Agnol *et al.*, 2016; Silva *et al.*, 2018; Paulitsch *et al.*, 2019). In India, many researchers reported different species (mostly from β-Rhizobia) associated with Mimosa (Metal *et al.*, 2003; Pandey *et al.*, 2005; Sinhga *et al.*, 2016). Sukna forest is a part of Eastern Himalayan in North Bengal, have a rich biodiversity of microbial flora. Sukna forest is geographically located at 88.3199°E longitude and 28.8065°N latitude in the north eastern part of India. The objectives of the present study were identification, morphological and biochemical characterization of β-rhizobia isolated from root nodules of *Mimosa pudica* and to study the potential of isolated strains as plant growth promoters.

Materials and methods

Collection of strains and isolation: Plants of *Mimosa pudica* were uprooted from the different parts of Sukna forest of North Bengal (India) (Fig. 1). Fresh and undamaged nodules were selected for rhizobia isolation. For surface sterilization, nodules were washed with sterile distilled water several times followed by 70% ethanol for 2 min and 4%(v/v) sodium hypochlorite solution for 3 min and final rinsing was done with sterile distilled water several times. Surface sterilized nodules were put on grease free sterile slide and crushed in a drop of sterile distilled water with the help of sterile glass rod and obtained milky suspension. A loopful of this milky suspension was streaked on yeast extract mannitol agar plates (YEMA) containing 0.0025% (w/v) congo red (Vincent, 1970). The YEMA plates were incubated at 28±2°C for 2-3 d and single pure colonies were obtained. Pure culture maintained in the YEMA slants and subjected to phenotypic and biochemical characterization. In order to compare the phenotypic and genotypic traits of the isolates with reference strains, *Burkholderia cepacia* MTCC-4684 were obtained from Institute of Microbial Technology (IMTECH), Chandigarh.

Morphology and biochemical characterization: Morphology of the colonies was characterized based on their color, odor, shape, appearance, colony diameter, margin and transparency (Dubey and Maheshwari, 2007). Motility test and Gram staining reaction were conducted to characterized isolates. Various biochemical test such as starch hydrolysis, catalase, ketolactase, glucose peptone agar, methylene blue, Hofer's alkaline, triple sugar iron, acid-alkaline production, nitrate reduction and carbohydrate utilization tests were performed using standard methods described by Somasegaran and Hoben (1994).

Physiological characterization: The isolates were grown on YEMA plates with 1, 2, and 3% (w/v) concentration of sodium chloride (NaCl) to check the resistance to salinity. Inoculated plates were incubated at 30°C for 3-4 d and examined for presence or absence of growth. The isolates were cultured on media containing different pH (5, 7 and 8) to perform pH tolerance test. For Temperature stress tolerance test, the isolates were incubated at different temperature ranges from 10°C to 40°C.

Plant growth promoting rhizobial abilities (PGPR): Phosphate solublization test: A loopful of fresh grown culture was spot inoculated on Pikovskaya's agar medium and plates were incubated at 30°C for 3-5 d. The presence or absences of clear transparent hallo zone around the colonies were observed (Pikovskaya, 1948).

Ammonia production test: Culture was inoculated in test tubes containing peptone water and after the incubation transparent yellow color was observed (Cappucino and Sherman, 1992).

IAA production test: Indole acetic acid (IAA) production test was followed as per Bric *et al.* (1991). YEM broth supplemented with 0.1% L-tryptophan was inoculated and incubated for 2 d at 200rpm at 30°C. After incubation culture were centrifuged at 6000x for 10 min at 4°C.

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Two mL supernatant was then mixed with 4 mL Salkowski reagent (1 mL of 0.5M $feCl_3$ in 50 mL 35% perchloric acid) and the absorbance of the resultant was recorded at 530 nm in visible spectrophotometer.

Siderophore production: Rhizobial isolates were spot inoculated on Chrome-azurol S (CAS) agar media plates and incubated 2-5 d at 30°C. Colonies were observed for orange halo colored zone formation.

Antagonistic study against fungal pathogen: Isolated rhizobial strains were used for antagonistic study against the fungal pathogen *Fusarium solani* obtained from Immuno-Phytopathology Laboratory, Dep. of Botany, North Bengal University. Pure culture of *Fusarium solani* was grown on PDA medium for 6-7 d. About 5 mm of mycelial disc from fully grown fungal culture was inoculated on one side of fresh petridish containing PDA medium while another side of petridish was streaked by rhizobial isolates 5 mm away from the fungal pathogen. Plates were kept for 7 d incubation at 30°C. The antifungal activity was calculated by measuring the resulting distance between the bacterial isolates and fungal pathogen. Test was repeated 3 times to avoid any error.

Root nodulation assay: Based on their performance in plant growth promoting ability tests, selected isolates were subjected to nodulation assay. Sterilized seeds of *Mimosa pudica* and were sown in pots containing N-free sterile soil (Koch's postulation). Pots were inoculated with the isolated rhizobial strains and after 4 months roots were observed with nodules. Colonies isolated from surface sterilized nodules were used for biochemical analysis.

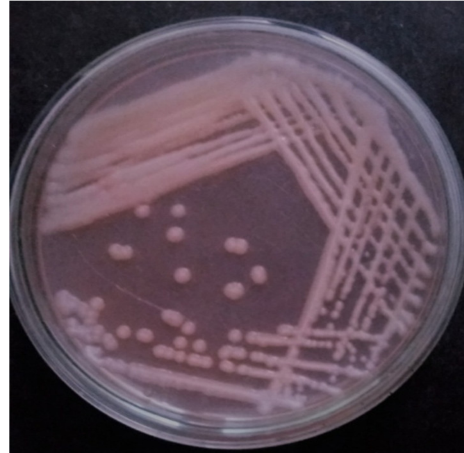
Molecular characterization of isolates

Genomic DNA extraction: The strains were incubated in YEMA plates at 30°C for 48 h to extract genomic DNA using standard protocol of William and Feil (2012) with minor modifications.

Amplification, sequencing and analysis of 16S rRNA gene: PCR amplification of 16S rRNA gene was carried out by universal primers 27f5'AGAGTTTGATCATGGCTCAG3' and 1492r5'ACGGATACCTTGTTTACGACTT3' (Weisburg *et al.*, 1991). Amplification was performed in a total volume of 50 µL containing 1 µL of genomic DNA (30 ng), 2.5 µL of dNTP (2.5 mM), 5 µL of PCR buffer (10 x), 1 µL of each primer , 0.5 µL of Taq polymerase (1.25 U) and 40 µL of nuclease free water. PCR conditions were followed as: initial denaturalization for 1 min at 94°C followed by 30 cycles of 5 min each at 95°C, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension for 7 min at 72°C. After that, a 5 µL aliquot of the PCR reaction was used to visualize the amplified products on a 2% agarose gel in TBE 1X buffer.

Fig. 2. Roots of *Mimosa pudica* with nodules.

Fig. 3. Isolates colony in YEMA with congo red.



The amplified PCR products were sequenced at National Centre for Microbial Resource, Pune, India. Alignment was done using the BioEdit Sequence Alignment program (Altschul *et al.*, 1990). The acquired sequences of isolate were compared with the related sequences obtained from GeneBank database with the BLAST program and aligned them by CLUSTAL W program (Thompson *et al.*, 1994). A phylogenetic tree was constructed using Molecular Evolutionary Genetic Analysis (MEGA) software (Tamura *et al.*, 2007).

Result and discussion

Rhizobia isolated from *Mimosa pudica* roots (Fig. 2) showed best growth on YEMA medium at pH 7.0 for 24-48 h incubation at 30°C (Fig. 3). Around 70% of rhizobial isolates colonies were found to be circular (2-3 mm in dia), convex, smooth, semi translucent, gummy, cremish in nature (Fig. 3; Table 1).

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Table 1. Morphological characterization of colonies grown in YEM media.

Isolate	Shape	Size (dia in mm)	Margin	Elevation	Color	Odor	Motility	Gram's Nature
SUK ND1	Circular	2-3	Entire	Convex	Creamish	Musky	Motile	Gram-ve
SUK ND2	Circular	2-3	Entire	Convex	Creamish	Musky	Motile	Gram-ve
SUK ND3	Circular	1-2	Entire	Convex	Creamish	Musky	Motile	Gram-ve
SUK ND4	Circular	2-3	Entire	Convex	Creamish	Musky	Motile	Gram-ve
SUK ND5	Circular	2-3	Entire	Raised	Creamish	Musky	Motile	Gram-ve
SUK ND6	Circular	1-2	Entire	Raised	Creamish	No odor	Motile	Gram-ve
SUK ND7	Circular	2-3	Entire	Convex	Transparent	Musky	Motile	Gram-ve
SUK ND8	Circular	2-3	Entire	Convex	Creamish	Musky	Motile	Gram-ve
SUK ND9	Circular	3-4	Entire	Convex	Creamish	Musky	Motile	Gram-ve
SUK ND10	Circular	2-3	Entire	Convex	Creamish	No odor	Motile	Gram-ve
B. cepacia	Circular	2-3	Entire	Convex	Creamish	-	Motile	Gram-ve

Table 2. Biochemical features of isolates.

Isolate	KT	NRT	GH	SH	GPA	Hofer's test	CAT	MB	TSI	Acid-Alkaline test
SUK ND1	-	+	+	+	+	-	-	+	-	Acid production
SUK ND2	-	+	+	+	+	-	-	+	RSYB	Acid production
SUK ND3	-	+	+	+	+	-	-	+	RSYB	Acid production
SUK ND4	-	+	+	+	+	-	-	+	-	Acid production
SUK ND5	-	+	-	+	+	-	-	-	-	Acid production
SUK ND6	-	+	-	-	+	-	+	+	-	Acid production
SUK ND7	-	-	+	+	-	-	+	+	RSYB	Acid production
SUK ND8	-	+	+	+	+	-	-	+	-	Acid production
SUK ND9	-	+	+	+	+	-	-	+	RSYB	Acid production
SUK ND10	-	+	-	+	-	-	-	-	RSYB	Acid production
B. cepacia	-	+	+	-	+	-	+	-	-	Acid production

GPA= Glucose peptone agar; MB= Methylene blue; SH= Starch hydrolysis; LA= Lactose assay; Cat= Catalase; GH= Gelatin hydrolysis; TSI= Triple sugar iron agar; NRT= Nitrate reduction test; KT= Ketolactase test; RSRB= Red stab red butt; RSYB= Red stab yellow butt.

Table 3. Phenotypic and carbon utilization tests.

Isolates	Growth at pH			Temperature tolerance (°C)				NaCl tolerance (%)			Carbon utilization test			
	5	7	8	10	20	30	40	1	2	3	Fru	suc	lac	xyl
SK ND1	+	+	+	-	+	+	-	+	+	-	+	+	-	-
SK ND2	-	+	+	-	+	+	-	+	+	-	+	+	-	-
SK ND 3	-	+	-	-	+	+	-	+	+	-	+	+	+	-
SK ND 4	-	+	+	-	+	+	-	+	+	-	+	+	+	-
SK ND5	-	+	-	-	-	+	-	+	-	-	+	+	-	-
SK ND6	-	+	-	-	-	+	-	+	-	-	+	+	-	-
SK ND7	-	+	-	-	-	+	-	+	-	-	+	+	-	-
SK ND8	+	+	+	-	+	+	-	+	+	-	+	+	+	-
SK ND9	-	+	+	-	+	+	-	+	+	-	+	+	+	-
SK ND10	-	+	-	-	-	+	-	+	-	-	+	+	-	-

Fru= fructose; suc= sucrose; lac= lactose; xyl= xylose.

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Table 4. Plant growth promoting ability tests.

Isolates	IAA (µg/mL)	PSE (%)	Ammonia production	Siderophore	Antagonistic activity
SK ND1	58	56.25	+	+	+
SK ND2	54	35.29	+	+	+
SK ND 3	59	50	+	+	+
SK ND 4	50	56.25	+	+	+
SK ND5	00	8	-	-	-
SK ND6	10	00	-	-	-
SK ND7	2	00	-	-	-
SK ND8	61	57.14	+	+	+
SK ND9	54	37.5	+	+	+
SK ND10	00	00	-	-	-

IAA= Indole acetic acid; PSE= Phosphate solublization efficiency.

The present study showed that all isolates grew well at pH 7 and a few of them grew at pH 5 and 8. In the salinity tolerance test, SK ND1, SKND2, SKND3, SKND4, SKND8 and SKND9 at 2% NaCl and all the isolates survived at 1% NaCl. The isolates showed no growth at 3% NaCl. Similar results were also reported by Pandey *et al.* (2005), the presence of *Burkholderia* sp. in the root nodule of *M. pudica* that effectively tolerated 1% NaCl. Additionally, the bacterial isolates showed maximum growth at 30°C while the growth decreased with the increase and decrease in temperature (Table 3). Salinity, temperature and soil pH plays an important role in limiting the growth of microorganisms in soil and on the basis of stress tolerance condition isolates could be used as effective inoculants in future. In present study, plant growth promoting ability test revealed that the some bacterial strains produced IAA, siderophore, ammonia and solubilized inorganic phosphate. IAA production was calculated from the regression equation of standard curve and the results were expressed as µg/mL. An orange halo zone around the colonies, indicating siderophores production observed in few strains. Pandey *et al.* (2005) reported similar results in rhizobia isolated from *M. pudica*. Present study showed that some isolates were capable of phosphate solubilization and it is determined by comparing the colony diameter of the strains with the diameter of the solubilization zone. Six isolates showed high phosphate solubilization efficiency and were capable of producing ammonia (Table 4). On inoculation of isolates in peptone broth, faint yellow turned to dark brown color indicated ammonia production. Similar observations were reported by Bhargava *et al.* (2016) in rhizobial isolates from *M. pudica*. It has been reported that different species of rhizobia can significantly reduce wilt and root rot disease of common bean and chickpea caused by *Fusarium solani* (Kucuk, 2013) acting as a biocontrol agent. The antagonistic activity of rhizobial isolates against the fungal pathogen *F. solani* was recorded after 7 d of incubation.

Fig. 4. Pure culture of *Fusarium solani.*

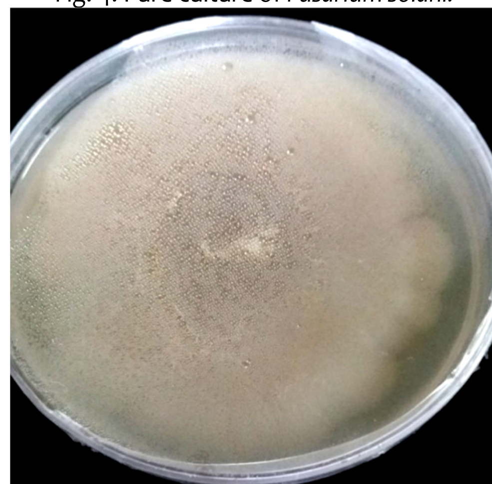

Fig. 5. Isolates showing antagonistic activity against *Fusarium solani.*

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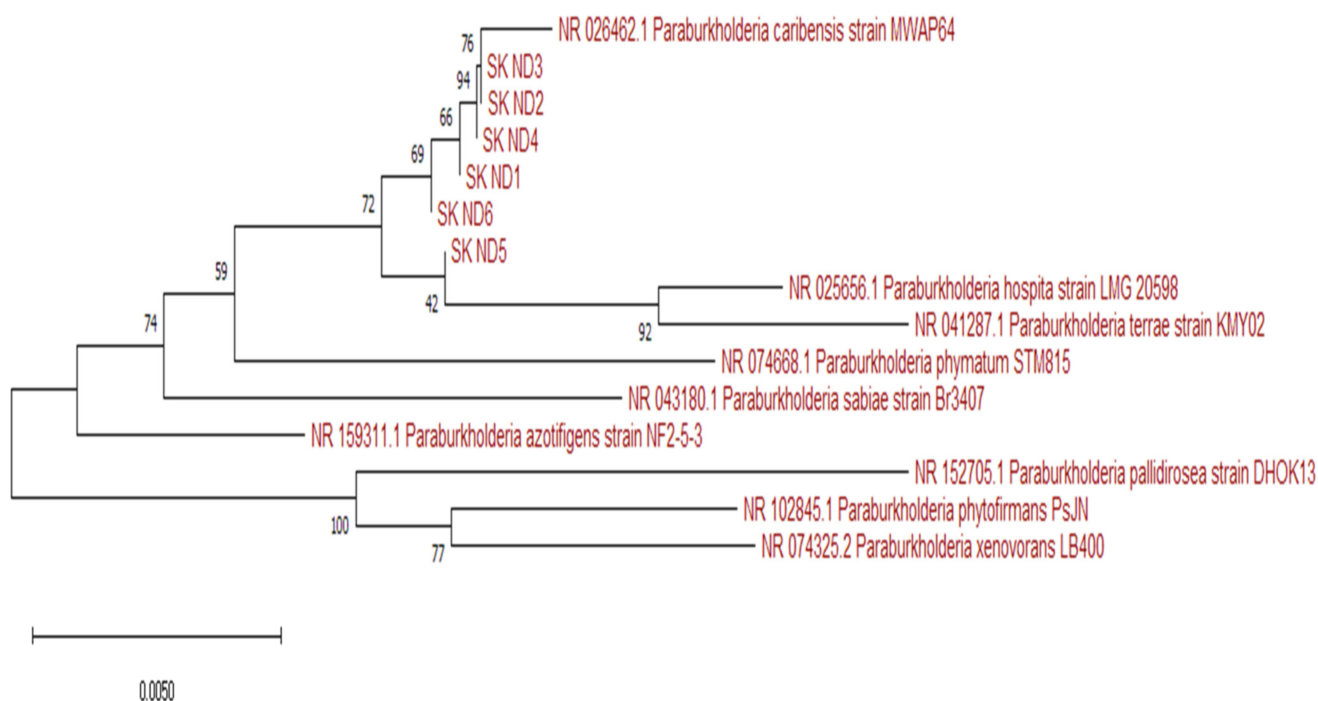
Table 5. GenBank accession numbers for partial 16S rRNA sequence of *Paraburkholdera.*

Strain	Isolates (location)	Sequence's Length (bp)	GenBank (Accession No.)	Maximum similarity (%) with other Sequence in GenBank with accession no
SK ND1	North S F	1389	MT774776	99.7% with *P.caribensis* MWAP64(T) - CP013102
SK ND2	South S F	1430	MT774777	99.7% with *P.caribensis* MWAP64(T) - CP013102
SK ND3	East S F	1493	MT774778	99.4% with *P.caribensis* MWAP64(T) - CP013102
SK ND4	West S F	1458	MT774779	99.7% with *P.caribensis* MWAP64(T) - CP013102
SK ND8	Centre S F	1252	MT774780	99.6% with *P.caribensis* MWAP64(T) - CP013102
SK ND9	Highest top S F	1189	MT774781	99.8% with *P.caribensis* MWAP64(T) - CP013102

SF: Sukna Forest.

Fig. 6. Phylogenetic (neighbor-joining) tree depicting the relationship of six isolates and 9 reference strains from NCBI database. GenBank accession numbers as are followed by their species names.



The results revealed that isolates SUKND1, SUKND2, SUKND3, SUKND4, SUKND8 and SUKND10 were shown outstanding antifungal activity against fungal pathogen (Fig. 4 and 5). Based on biochemical characteristics, antagonistic activity and plant growth promoting potential, six isolates viz. SKND1, SKND2, SKND3, SKND4, SKND8 and SKND9 were selected for nodulation assay and molecular characterization. All the selected isolates were capable to nodulate *Mimosa pudica* seedlings grown modified nitrogen free medium. Nodules were light brown color, oval shaped, and 1 to 1.5 in dia. The 16S rRNA gene of the isolates SKND1, SKND2, SKND3, SKND4, SKND8 and SKDN9 were amplified and sequenced for identification.

DNA sequences were subjected the BLASTN to identify the most similar sequences available in the database and revealed that all the isolates have almost 99% similarity with *Paraburkholderia caribensis*. Sequences were submitted to GenBank for obtaining GenBank accession numbers (Table 5). Two strains microbial culture out of six were deposited in National Centre for Microbial Resource, Pune (Accession number- MCC 3533 and MCC 3534), available to public access. Nine *paraburkholderia* strains sequences were retrieved from NCBI database and evolutionary history was analyzed using Neighbour-Joining method. A phylogram identified six strains as a species of paraburkholderia with a 100% bootstrap support (Fig. 6).

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Conclusion

Phenotypic characterization of isolates from root nodules of *Mimosa pudica* indicated that they are tolerant to abiotic conditions including salinity and temperature stress. Isolated strains were identified as highly effective at plant growth promoting abilities and can significantly reduce wilt and root rot disease caused by *Fusarium solani.* Isolates with such characters could be used as effective biofertilizers for sustainable agriculture development. In the present study, it revealed that bacterial strain isolated from different regions of Sukna forest belonging to the species *Paraburkholderia caribensis* was the predominant symbionts of *Mimosa pudica.*

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