



Diverse key nitrogen cycling genes *nifH*, *nirS* and *nosZ* associated with Pichavaram mangrove rhizospheres as revealed by culture-dependent and culture-independent analyses

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Abstract

Mangroves are highly productive unique ecosystems harboring diverse unexplored microbial communities that play crucial roles in nutrient cycling as well as in maintaining ecosystem services. The mangrove-associated microbial communities transform the dead vegetation into nutrient sources of nitrogen, phosphorus, potash, etc. To understand the genetic and functional diversity of the bacterial communities involved in nitrogen cycling of this ecosystem, this study explored the diversity and distribution of both the nitrogen fixers and denitrifiers associated with the rhizospheres of *Avicennia marina*, *Rhizophora mucronata*, *Suaeda maritima*, and *Salicornia brachiata* of the Pichavaram mangroves. A combination of both culturable and unculturable (PCR-DGGE) approaches was adopted to explore the bacterial communities involved in nitrogen fixation by targeting the *nifH* genes, and the denitrifiers were explored by targeting the *nirS* and *nosZ* genes. Across the rhizospheres, Gammaproteobacteria was found to be predominant representing both nitrogen fixers and denitrifiers as revealed by culturable and unculturable analyses. Sequence analysis of soil *nifH*, *nirS* and *nosZ* genes clustered to unculturable, with few groups clustering with culturable groups, viz., *Pseudomonas* sp. and *Halomonas* sp. A total of 16 different culturable genera were isolated and characterized in this study. Other phyla like Firmicutes and Actinobacteria were also observed. The PCR-DGGE analysis also revealed the presence of 29 novel *nifH* sequences that were not reported earlier. Thus, the mangrove ecosystems serve as potential source for identifying unexplored novel microbial communities that contribute to nutrient cycling.

Keywords PCR-DGGE · Nitrogen fixation · Denitrification · Gammaproteobacteria · BOX-PCR

Introduction

Nitrogen is one of the most important nutrients in all ecosystems; the nitrogen cycle mediated by microbes is a very complex process that involves the transformation of nitrogen to different forms through nitrogen fixation, nitrification, denitrification, ammonification, anaerobic ammonium oxidation, and dissimilatory nitrate reduction to ammonium

(Purvaja et al. 2008). Genomics and high-throughput sequencing methods provide a broader perspective on the diverse microbial communities and their functional genes involved in the nitrogen cycling process (Zhang et al. 2017).

The mangrove ecosystem located between terrestrial and marine interface environments along the tropical and subtropical coastline is frequently inundated by floods and high tides (Holguin et al. 2001; Giri et al. 2011), but play a major role in protecting the coasts in the tropical and subtropical regions. This ecosystem is partially anaerobically coupled with high salinity and low oxido-reductive potential; the microbiota associated with mangroves is represented by a combination of terrestrial, freshwater, and marine microorganisms that are crucial to the biogeochemical productivity (Vazquez et al. 2000). The bacterial and archaeal groups inhabiting this ecosystem play a major role in nutrient transformation, ecological and biogeochemical functions (Cao et al. 2011), and are influenced by salinity (Silveira et al. 2011), organic carbon (Dunaj et al. 2012), nitrogen content

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(Carriero et al. 2012), climate, and chemical substances (Bragazza et al. 2015), which in turn determine the diversity, distribution, and function of the microbial communities in this ecosystem. The mangroves serve as hot spots for the discovery of novel microbes with novel ecological functions (Rameshkumar et al. 2014; Raju et al. 2016).

Nitrogen fixation and denitrification have been reported in a phylogenetically diverse group of bacteria and archaea; their diversity and distribution can be determined by targeting functional marker genes such as *nifH*, *nirS*, and *nosZ* (Jenkins and Zehr 2008). A diverse group of bacteria and archaea harboring *nifH/nirS* involved in nitrogen fixation and denitrification has been reported from the estuarine ecosystems. Since most of these remain unculturable, advanced molecular tools have been employed to understand the diversity, function, and distribution of these microbial groups (Ren et al. 2018).

Nitrogen fixation, a process where gaseous nitrogen (N_2) is converted to biologically available forms such as ammonia (NH_3) by diazotrophs is considered to be the major source of combined nitrogen input in mangrove forest habitats. Thus, the high productivity of mangrove ecosystems might be partially attributable to the high rate of biological nitrogen-fixing activity of free-living diazotrophs in the rhizosphere of mangroves as well as the sediments (Holguin et al. 2001).

Denitrification, a functional trait distributed among a taxonomically diverse group of microbes, (Zhang et al. 2013) is primarily a bacterial respiratory process regulated by four different enzymatic steps and catalyzed by four Metalloproteins such as nitrate reductases, nitrite reductases (*nir*), nitric oxide reductase and nitrous oxide reductases (*nos*) (Braker et al. 2000). Denitrification is reported in a wide range of heterotrophic (e.g., *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, and *Paracoccus denitrificans*) and autotrophic bacterial communities (e.g., *Thiobacillus denitrificans*) belonging to the phylum Proteobacteria (Green et al. 2010). However, the microbe-mediated denitrification also acts as a sink by removing the excess anthropogenic N input; thus, it may prevent the transport of excess nitrogen to the estuarine and coastal systems which poses a serious threat to these ecosystems (Woodward et al. 2009).

With the culture-based analysis, only a minimal proportion of the microbial population can be retrieved from any sample, leaving the rest undetected or uncultured. Hence, studies on microbial community assessment, using a wide range of techniques such as classical cultivation procedure, fingerprinting, clone libraries, and next-generation sequencing (Andreote et al. 2012; Zhang et al. 2017) have revealed the extensive microbial diversity and its function that were not detected earlier. Compared to other techniques like SSCP or t-RFLP, DGGE is one of the fingerprinting approaches that have been designed to study microbial communities which cannot be attained with the cultivable fraction

represented by < 1% of the total number of prokaryotic species present in a given sample as reproducibility is the main disadvantage in the later techniques. DGGE has been used to explore communities from a wide range of ecosystems and also the microbes that are involved in the biogeochemical cycling of an ecosystem (Rastogi et al. 2010).

This study mainly focused on accessing the diversity and distribution of bacterial communities associated with mangrove rhizospheres that contribute to nitrogen fixation by targeting the *nifH* genes and the denitrifiers harboring *nirS* and *nosZ* genes by both culture-dependent and independent approaches. Therefore, exploring the diversity and distribution of rhizosphere-associated diazotrophs and denitrifying microbial communities that drive the nitrogen cycle is essential to understand the biogeochemical cycling of nitrogen.

Materials and methods

Site description and sampling

The study site Pichavaram mangroves are located on the southeast coast of India near Chidambaram, situated about 250 km away from Chennai. It is an estuary located in between Coleroon and Velar estuary which covers a total area of 1350 ha colonized by true mangrove species and some halophytic plants. The rhizosphere soil samples of *Avicennia marina*, *Rhizophora mucronata*, *Suaeda maritima*, and *Salicornia brachiata* were collected randomly using a soil core, and transferred to sterile polyethylene bags, and transported to the laboratory on ice within 6 h. The geographical location of each sampling site was recorded using a global positioning system instrument (GPS) (GARMIN Etrex, Taiwan). A total of 10 samples were collected from the rhizosphere region with a minimum distance range of 2 km of which 3 samples were from *A. marina*, 3 from *R. mucronata*, 2 from the intersecting region of *A. marina*, and *R. mucronata*, 1 sample each from *S. maritima* and *S. brachiata*. The samples were stored at $-80\text{ }^\circ\text{C}$ for deoxyribonucleic acid (DNA) extraction.

Soil DNA extraction and PCR amplification of nitrogen cycling genes

Total genomic DNA was extracted from each rhizosphere soil sample by CTAB-SDS method as described by Ghosh et al. (2010) and purified using MO-BIO DNA (MO BIO Laboratories, USA) clean-up kit as per manufacturers guidelines and stored at $-20\text{ }^\circ\text{C}$ for further analysis. The targeted genes amplified using gene-specific primers were given in the below section and annealing conditions are given in Table 1. Amplification was verified by agarose gel electrophoresis and was subjected to DGGE analysis.

Table 1 Primers and amplification conditions for culturable/unculturable bacteria

	Primers	Nucleotide sequences (5'→3')	Length	Amplification conditions
Nitrogen fixers	PolF*	TGCGAYCCSAARGCBGACTC	390 bp	Initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min; annealing at 52 °C for 1 min; primer extension at 72 °C for 1 min; and final extension at 72 °C for 10 min
	PolR	ATSGCCATCATYTCRCCGGA		
Denitrifiers	nirS 1Fd	CCTAY(C/T)TGGCCGCCR(A/G)CAR(A/G)T	890 bp	Initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min; annealing at 58 °C for 1 min; primer extension at 72 °C for 1 min and final extension at 72 °C for 10 min
	nirS 6Rd	CGTTGAACTTR(A/G)CCGGT		
	cd3aFGC*	GT(C/G)AACGT(C/G)AAGGA(A/G) AC(C/G)	425 bp	Initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min; annealing at 59 °C for 1 min; primer extension at 72 °C for 1 min and final extension at 72 °C for 10 min
	R3cd	GG GA(C/G)TTCGG(A/G)TG(C/G)GTCTTGA		
	nosZ 661F	CGGCTGGGGGCTGACCAA	1100 bp	Initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min; annealing at 57 °C for 1 min; primer extension at 72 °C for 1 min and final extension at 72 °C for 10 min
	nosZ1773R	AACGAV(A/C/G)CAGY(T/C)TGATCGAY(T/C) AT		
nosZFGC*	CG(C/T)TGTTT(A/C)TCGACAGCCA	453 bp	Initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min; annealing at 57 °C for 1 min; primer extension at 72 °C for 1 min and final extension at 72 °C for 10 min	
nosZ1662r	CGC(G/A)A(C/G)GGCAA(G/C)AAGGT(G/C) CG			
BOX A1R	5'CTACGGCAAGGCGACGCTGACG3'		Initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 53 °C for 1 min; extension at 63 °C for 8 min and final extension at 72 °C for 8 min	

For DGGE analysis 40 bp gc clamp is added to 5' end of forward sequence marked in *

PCR-DGGE analysis

About 45 µl of the amplified products were loaded onto DGGE gel in the Dcode™ mutation detection system (Bio-Rad, Laboratories, CA, USA). The electrophoresis was carried out at a constant temperature of 60 °C for 17 h, in 8% polyacrylamide gel (100% denaturant, 7 M urea, and 40% (vol/vol) formamide) with a 50 to 60% denaturant gradient and stained with SYBR gold nucleic acid and kept in dark for 20 min. The gel was then rinsed with double sterilized dist. water and the gel images were documented using a UV illuminator (Gel-doc XR+, Bio-Rad laboratories, CA, USA). The digitized gel images were analyzed using Bio-Rad fingerprinting II software and the position of the bands were recorded and the variable bands were then eluted from the gel by marking the band position with a sterile scalpel and transferred to sterile microcentrifuge tubes with TE buffer (pH 8.0) and incubated overnight at 4 °C. To reconfirm the purity and position of the bands, 6 µl of the eluted bands was used as a template, with the same combination of DGGE primers but without GC clamps, the total reaction volume was made up to 50 µl, reamplified and run in the same gradient DGGE and its position was confirmed and the products were purified.

Cloning and sequencing of DGGE bands

The purified PCR products were then ligated into pTZ57R/T cloning vector (InsTAclone PCR cloning kit, Fermentas, Thermo Scientific) and the ligation mixture was prepared as per the manufacturer's instruction with slight modification, which contained 1 µl of 10 X ligation buffer, 5 µl of template DNA, 1 µl of vector, 1 µl ligase enzyme and 2 µl of nuclease-free water. The mixture was then incubated at 4 °C overnight for efficient ligation and transformed into competent cells of *E. Coli* strain XL1-Blue (Novagen, Madison, WI, USA). Plasmids were extracted using the Favorgen plasmid DNA extraction Miniprep kit (Favorgen, Taiwan) as per manufacturer instruction and the positive clones were sequenced. All the sequences were analyzed through BLAST-N for determining the taxonomic identity and tBLAST-X for determining the protein identity. Phylogenetic trees were constructed by the neighbor-joining method using Mega 5.0.

Culturable analysis of nitrogen fixers and denitrifiers

About 5 g of the individual rhizosphere soil samples were transferred into test tubes containing 50 ml of sterile dist.

water and vortexed for about 30 min and serially diluted up to 10^8 , about 0.1 ml was spread plated onto LB agar, Nitrogen free medium, and BTB agar plates (Takaya et al. 2003). The plates were incubated for 3–4 days at room temperature and based on colony morphology, individual colonies were picked, and maintained as pure cultures in their respective media and also stored in 25% glycerol stocks at $-80\text{ }^\circ\text{C}$.

Genomic DNA isolation of culturable bacteria

Genomic DNA was extracted from overnight grown cultures in 10 ml LB broth incubated at $32\text{ }^\circ\text{C}$ for 24 h. The isolated DNA has been subjected to PCR-based screening for the presence of nitrogen fixers and denitrifiers (Table 1).

Screening for *nifH*, *nirS*, and *nosZ* harboring bacteria

The *nifH* gene fragments were amplified using primers as described by Poly et al. (2001) and the denitrifiers, *nirS*, and *nosZ* gene was amplified using primers described by Braker et al. (2000). All the PCRs were carried out in BioRad T100 thermal cycler. Primers and PCR conditions are given in Table 1.

Culturable diversity analysis using BOX PCR fingerprinting

The genetic diversity among culturable nitrogen-fixing and denitrifiers was determined by BOX-PCR profiling using BOX A1R primers (BenHaim et al. 2003). Primer and amplification conditions are given in Table 1. About 5 μl of the PCR products were run in EtBr stained 2% agarose gel in 0.5X TBE buffer at a constant voltage of 80 V for 3–4 h. The BOX-PCR profiles were visualized under UV illuminator, followed by a digital image capturing using the BioRad gel documentation system. Normalization, recognition, and band assignment were made using Fingerprinting II software (BioRad, USA) by Dice coefficient with an optimum range of 0.5%. The cluster analysis of similarity matrices was performed by Unweighted Pair Group with Mathematical Average (UPGMA) for dendrogram analysis.

Sequencing and data analysis of culturable bacteria

The universal bacterial primers 27f and 1492r were used to amplify culturable bacterial 16S rDNA genes and sequenced by Eurofins India Pvt. Ltd. The sequence similarities were compared in EzTaxon databases (Chun et al. 2007). The phylogenetic trees were constructed, using MEGA 5.0 (Tamura et al. 2011) to determine the taxonomic affiliation.

Data analysis

Digitized images of DGGE fingerprint were used to quantify diversity by using quantity one software which detects bands and quantifies the relative concentrations of amplified bands from cumulative pixel intensities within a given lane. The Shannon diversity index was calculated from the number of bands and relative intensities of bands present in each lane. To evaluate the correlation between the diversity of *nifH*, *nirS*, and *nosZ* genes among the different rhizosphere regions, multivariate principal component analysis (PCA) was performed from the data obtained from the DGGE patterns, based on band intensity and position, and were analyzed by adopting PCA. All the values were log-transformed before subjecting to analysis and PCA was performed using PAST 3.0. The similarities between the DGGE profiles were displayed graphically as a dendrogram based on UPGMA algorithms (unweighted pair group method with arithmetic averages).

Nucleotide sequence and accession numbers

The sequence data obtained in this study were deposited in NCBI gene bank under the following accession number—Culturable nitrogen fixers 16S rDNA: KU131229-131270; and denitrifiers 16S rDNA KU131271-131297. Unculturable *nifH*, *nirS* and *nosZ*: KY204253-204281, KY204282-204312, KY204313-204333.

Results

Soil characteristics

The GPS position along with the average of physicochemical properties of the ten rhizosphere soils of Pichavaram are shown in Tables 2 and 3. The soil was found to be clayey and pH was 7.2, the organic carbon (OC) was $<0.78\%$ and the organic matter 1.34%. The total nitrogen content was 916 mg/kg, while available nitrogen was 173 mg/kg which was lower than the available K (6434 mg/kg) in the present study. Available P, Z, Cu, Mn, Mo, and B were found to be below the detection limit (BDL). It was found that the available K was the most abundant macronutrient with 6434 mg/kg soil in Pichavaram (Table 3).

Culturable bacteria from mangroves

A total of 579 culturable bacterial isolates with different colony morphology were selected and screened for nitrogen fixers and denitrifiers. All isolates were maintained in LB agar for further analysis and stored as glycerol stock in $-80\text{ }^\circ\text{C}$.

Table 2 GPS points of samples

Sample no.	Plant species	GPS position
1	<i>Avicennia marina</i>	N 11,25,52.0 and E 079,47,30.9
2	<i>Avicennia marina</i>	N 11,25,35.9 and E 079,48,2.08
3	<i>Avicennia marina</i>	N 11,25,08.7 and E 079,48,14.2
4	<i>Rhizophora mucronata</i>	N 11,25,19.4 and E 079, 48,13.9
5	<i>Rhizophora mucronata</i>	N 11,25,0.79 and E 079,48,0.79
6	<i>Rhizophora mucronata</i>	N 11,25,52.5 and E 079,47,53.8
7	<i>Avicennia marina</i> and <i>Rhizophora mucronata</i>	N 11,25,52.2 and E 079,47,59.5
8	<i>Avicennia marina</i> and <i>Rhizophora mucronata</i>	N 11,26,07.6 and E 079,47,11.8
9	<i>Suaeda maritima</i>	N 11,25,29.0 and E 079,48,09.9
10	<i>Salicornia brachiata</i>	N 11, 24,59.1 and E 079,48,08.9

Table 3 Soil analysis of Pichavaram mangrove rhizosphere soils

S. no.	Test/parameter	Method	Results	Unit
1	Potassium (as K)	AOAC 990.08:2005	6434	mg/kg
2	Phosphorus (as P)	AOAC 990.08:2005	187	mg/kg
3	Nitrogen as N	IS10158:1982	916	mg/kg
4	Available phosphorus as P	Olsen's method	BDL(DL:10)	mg/kg
5	Total organic carbon	Walkely and Black, 1934	0.78	%
6	Total organic matter	Walkely and Black, 1934	1.34	%
7	Available Nitrogen as N	Alkaline permanganate method	173	mg/kg
8	Cation exchange capacity	USEPA 9081	39.3	mg/kg
9	Available zinc as Zn	Lindsay and Norvell (1978) by ICPOES	BDL(DL:10)	mg/kg
10	Available copper as Cu	Lindsay and Norvell (1978) by ICPOES	BDL(DL:10)	mg/kg
11	Available Magnesium as Mg	Lindsay and Norvell (1978) by ICPOES	18	mg/kg
12	Available Boron as B	Berger and Truog (1939) by ICPOES	BDL(DL:10)	mg/kg
13	Available Molybdenum as Mo	Ammonium oxylate extraction by ICPOES	BDL(DL:10)	mg/kg
14	Soil texture- clayey*			

BDL below detection limit

*The Mangrove Decade and Beyond: Activities, Lessons and Challenges in Mangrove Conservation and Management, MSSRF, Chennai, (2002)

Culture-independent analysis of *nifH* gene diversity (PCR-DGGE)

The DGGE profiles of *nifH* genes of all the 10 samples from 4 plant rhizospheres and an intermediate rhizospheric area showed varied banding patterns with a total of 10–15 bands per lane (Fig. 1). The profiles represented rich diversity in all the rhizosphere samples except *S. maritima* rhizosphere which had only 3–5 bands, indicating a low level of diversity of the *nifH* gene associated with this rhizosphere. A total of 29 DGGE ribotypes for *nifH* (Fig. 1) were eluted and assigned a unique number with a prefix MSSRF, i.e., MSSRF 1H to MSSRF 29H.

Cluster analysis of *nifH* DGGE ribotypes

The DGGE ribotypes of *nifH* gene formed four major clusters: (i) cluster A represented *nifH* ribotypes of *A. marina*,

S. maritima, and *R. mucronata* rhizosphere; (ii) cluster B represented *nifH* ribotypes of *A. marina* and *R. mucronata* rhizospheres, their intersecting region and *S. brachiata* rhizosphere; (iii) cluster C represented *nifH* ribotypes of *R. mucronata*; (iv) intersecting region of both *A. marina* and *R. mucronata* at 60% confidence level with considerable variation observed among different rhizosphere samples (Fig. 1b.)

Phylogenies of *nifH* gene sequences

The taxonomic identification of nitrogen-fixing bacterium that represents the unique DGGE bands is summarized in Table (S1). BLAST-N analysis of 29 sequences revealed that 10 sequences fell in the range of 80–89% similarity values and 19 sequences fell within the range of 90–99% and were similar to the *nifH* gene of uncultured organisms reported from various ecosystems. However, conversion of nucleotide to protein sequence analysis revealed that the majority of

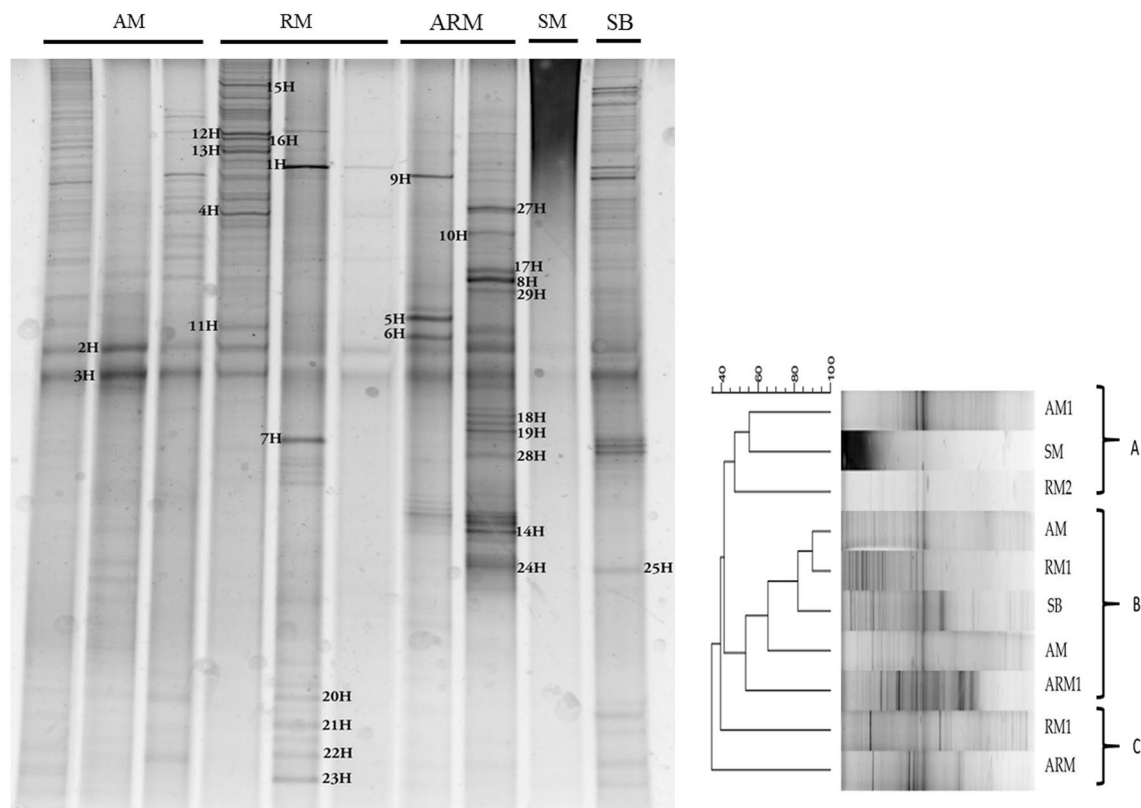


Fig. 1 a DGGE analysis of *nifH* gene. b Cluster analysis of DGGE profile nitrogen fixers by UPGMA algorithm (AM- *Avicennia marina*; RM- *Rhizophora mucronata*; ARM—*A. marina* and *R. mucronata* intersecting region; SM- *Suaeda maritima*; SB- *Salicornia brachiata*)

the sequences fell between 94 and 100% with similarity to the known *nifH* sequences of various environmental origins especially from saline soil and marine sediments. This indicates that the mangrove rhizosphere region harbors nitrogen-fixing bacterial communities similar to saline and marine environments. Further phylogenetic analysis of the *nifH* gene formed two major clusters with 11 subclusters indicating the presence of diverse *nifH* genes in this ecosystem. All the sequences in cluster 1 represented the sequences from marine sediments, saltmarsh, high and low saline soils, and sea sediments while cluster 2 showed similarity to sequences from rhizosphere soil of paddy and other terrestrial ecosystems (Fig. S1).

Diversity of culturable nitrogen-fixing bacteria

Nearly 52 strains formed pellicle in nitrogen-free medium and showed positive amplification for *nifH* gene with an amplicon size of 360 bp compared to *Ciceribacter lividus* MSSRFBL1^T used as a positive control. The BOX-PCR fingerprinting of the 52 strains showed genetic variation and formed 23 clades at an 80% confidence level (Fig. 2a). BOX-PCR based analysis has been widely recognized as one of the most common tools for determining microbial

diversity particularly between closely related groups (Ikeda et al. 2013).

Taxonomic classification of bacterial isolates harboring *nifH* gene

PCR-based 16S rRNA amplification and sequencing analysis of 1390–1410 bp of the amplified product of the culturable nitrogen fixers assigned the taxonomic identification up to generic and species level. The EzTaxon analysis of 16S rRNA gene sequences of the positive strains was compared with available sequences of the type strains in the database and was assigned the respective taxonomic position. The positive nitrogen fixers predominantly belonged to Gammaproteobacteria particularly the genus *Vibrio* (31%) comprising of seven species, i.e., *V. plantisponsor* MSSRF 40^T, *V. alginolyticus* NBRC 15630^T, *V. azureus* NBRC 104587^T, *V. diabolicus* HE800^T, *V. natrigenes* DSM 759^T, *V. parahemolyticus* NBRC 12711^T, and *V. neocaledonicus* NC470^T; followed by *Mangrovibacter* (12%) belonging to *M. plantisponsor* MSSRF 40^T, *Klebsiella* (12%) comprising of *K. pneumoniae* (DSM 30104^T), *Serratia* (6%) belonging to *S. marcescens* (KRED^T), and *Catenococcus thiocycli* DSM

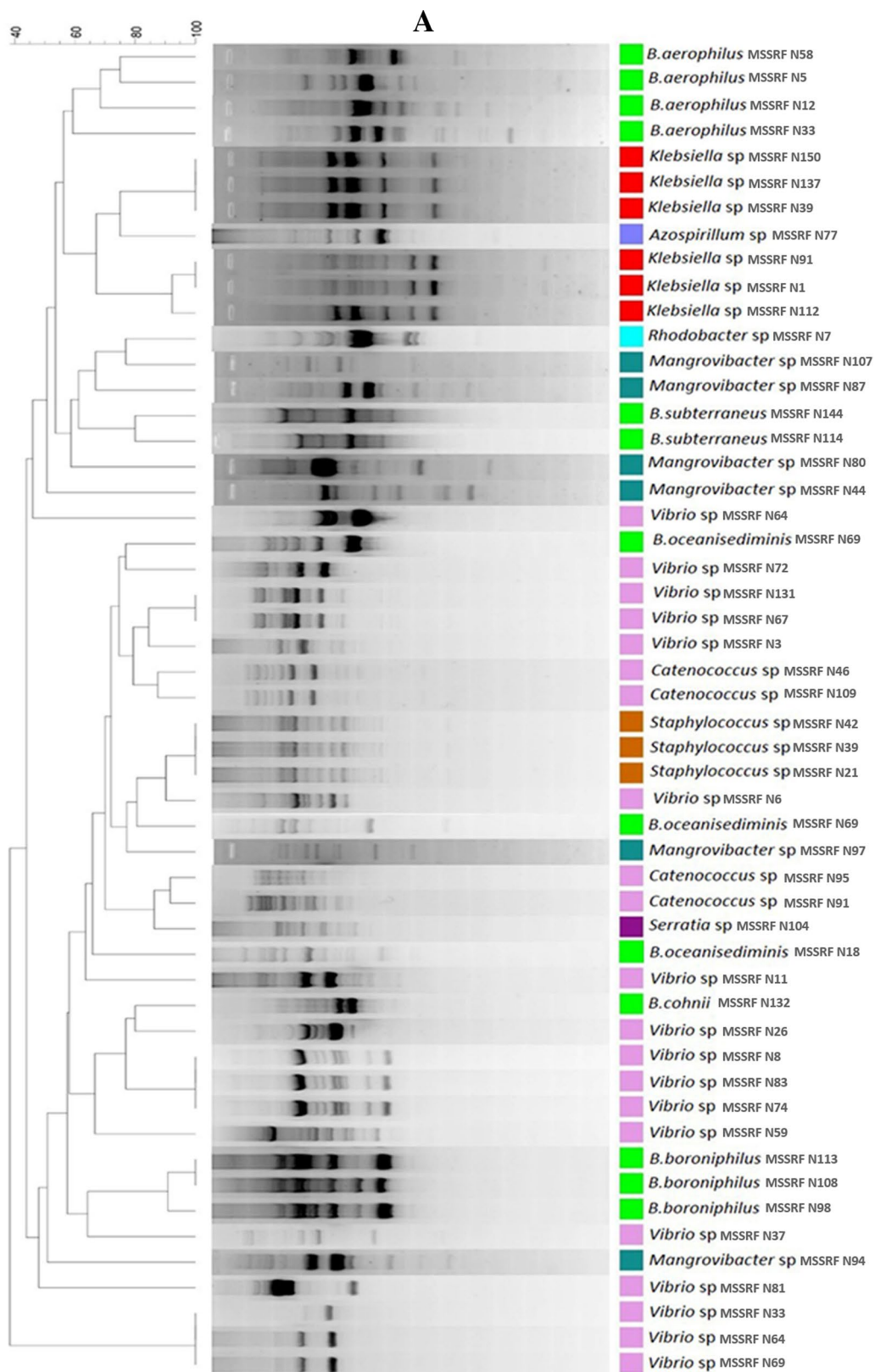


Fig. 2 a BOX-PCR Cluster analysis of culturable Nitrogen fixers based on Dice coefficient and dendrogram construction by UPGMA algorithm. **b** Neighbor joining analysis of culturable Nitrogen fixers

based on 16S rDNA analysis. A bootstrap value of 1000 replicates have been carried out for analysis

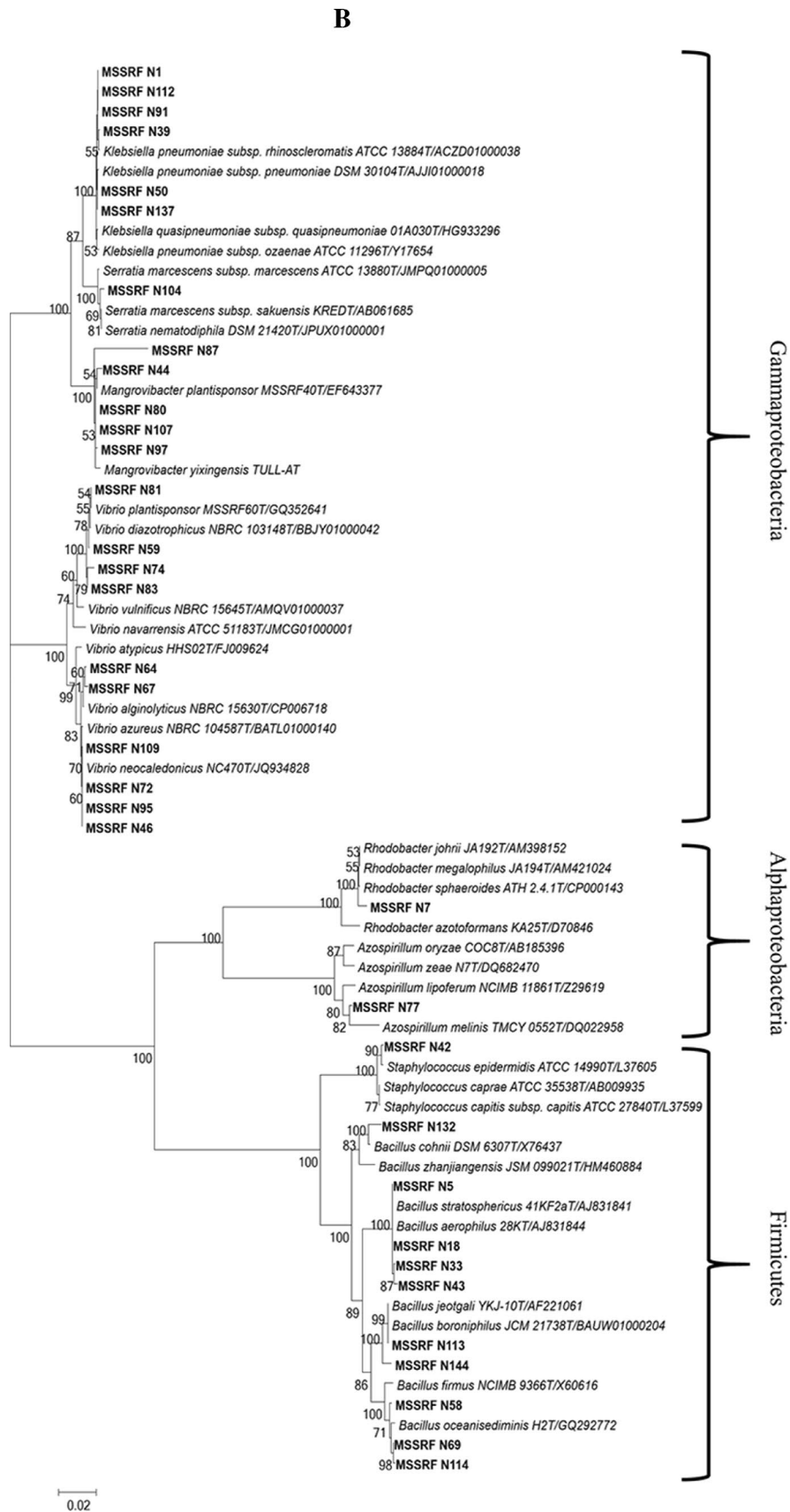


Fig. 2 (continued)

9165^T; the Alphaproteobacteria group was represented by *Rhodobacter johrii* JA192^T (2%), *Azospirillum lipoferum* NCIMB118161^T (2%) (8%) (Fig. 2b). The second-largest phylum was Firmicutes represented by *Bacillus* (21%) comprising of four species such as *B. aerophilus* 28K^T, *B. oceanisediminis* H2^T, *B. subterraneus* DSM 13966^T and *B. boroniphilus* JCM 21738^T; *Staphylococcus* (8%) comprising of *S. epidermis* (ATCC14990^T).

Culture-dependent and culture-independent analyses of denitrifying bacteria

DGGE analysis of *nirS* and *nosZ* genes

In this study, *nirS* and *nosZ* genes were used as a molecular marker to determine the distribution and diversity of culturable and unculturable denitrifying populations of mangrove rhizosphere. A total of 31 DGGE ribotypes for *cdnirS* coding nitrite reductase (Fig. 3a) and 21 DGGE ribotypes for *nosZ* gene coding nitrous oxide reductase gene (Fig. 4a) were eluted and assigned a unique number from MSSRF CD1 to MSSRF CD31 for *cdnirS* gene and MSSRF Z1 to MSSRF Z21 for *nosZ* gene.

Cluster analysis of *cdnirS* and *nosZ* gene ribotypes

At 60% confidence level, both *nirS* (Fig. 3b) and *nosZ* (Fig. 4b) genes formed five and four major clusters respectively, with a high degree of variation among the rhizosphere regions. Cluster A represented *nirS* genes from *A. marina*, *R. mucronata*, and *S. maritima* rhizosphere, while cluster B represents *nirS* genes from *A. marina* and *R. mucronata*, whereas clusters C, D, and E comprised ribotypes of intersecting region and *S. brachiata*. But the cluster analysis of the *nosZ* gene exhibited a unique pattern with the individual rhizosphere region forming a single cluster, e.g., cluster A comprised of *nosZ* ribotypes from *A. marina* and its intersecting region, cluster B represented *nosZ* ribotypes of *R. mucronata*, with an outward cluster of samples from both *S. maritima* and *S. brachiata*.

Phylogenies of *nirS* and *nosZ* gene sequences

The unique *nirS* sequences recovered from the mangrove rhizosphere shared 94–100% identities with known GenBank sequences. After translation, the corresponding protein sequences shared 75–100% identities to the closest matched *nirS* sequences detected from variety of marine

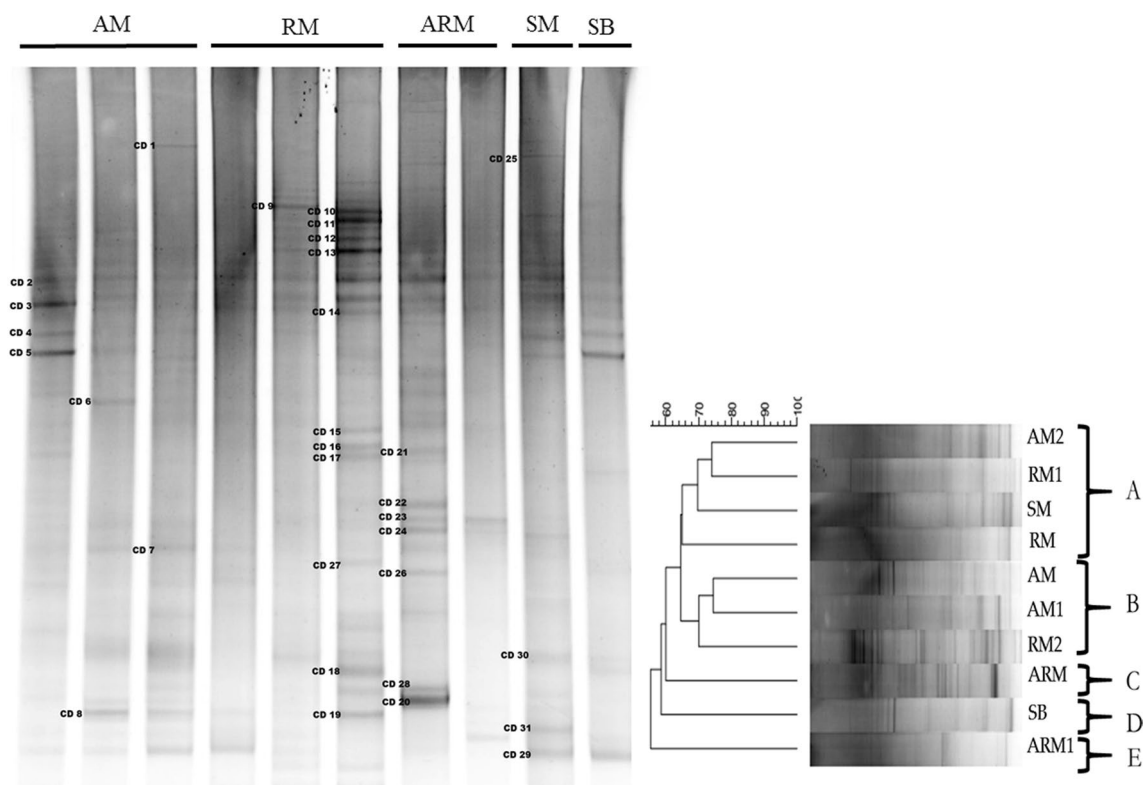


Fig. 3 a DGGE analysis of *nirS* gene. b Cluster analysis of DGGE profile *cdnirS* community by UPGMA algorithm (AM—*Avicennia marina*; RM—*Rhizophora mucronata*; ARM—*A.marina* & *R.mucronata* intersecting region; SM—*Suaeda maritima*; SB—*Salicornia brachiata*)

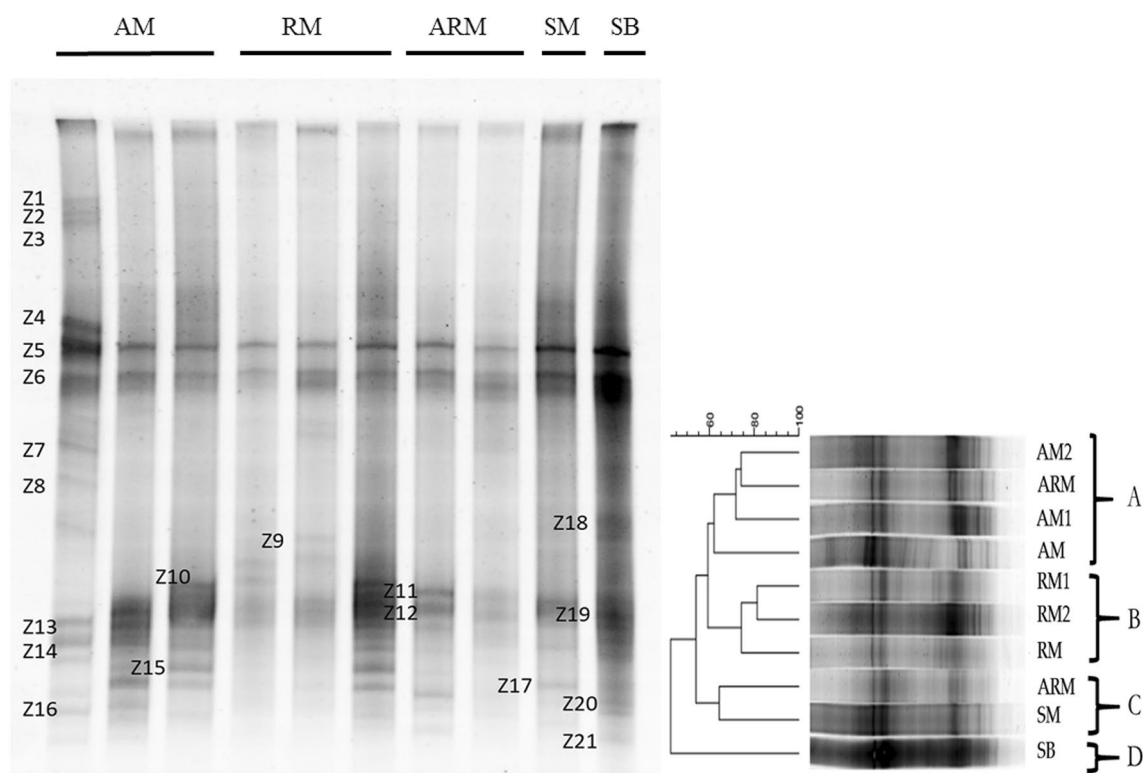


Fig. 4 **a** DGGE analysis of *nosZ* gene. **b** Cluster analysis of DGGE profile *nosZ* community by UPGMA algorithm (AM—*Avicennia marina*; RM—*Rhizophora mucronata*; ARM—*A.marina* & *R.mucronata* intersecting region; SM—*Suaeda maritima*; SB—*Salicornia brachiata*)

environments, saltmarsh sediment (cd19, cd23), Solar saltern (cd25), sludge (cd7, cd8), Agriculture soil (cd26), coastal sediments (cd2, cd5, cd11, cd22), sediments (cd14, cd18), estuary (cd12, cd16), soil (cd24, cd31) and landfill bioreactor, respectively (cd1, cd3, cd28), all of which showed similarity to uncultured nitrite reductase coding genes. However, the majority of the *nirS* (28 bands) sequences did not match with culturable denitrifiers and showed similarity to uncultured *nirS* gene sequences. Only 3 bands, namely CD29 showed 90% similarity to nitrite reductase gene of *Pseudomonas*, and two other bands CD25 and CD31 showed 96% and 92% similarity to nitrite reductase gene of *Halomonas nitroreducens* LMG 24185^T and *Halomonas cernia* LMG 24145^T strains, respectively (Fig. S2 and Table S2).

The DGGE ribotypes of the nitrous oxide reductase gene (*nosZ*) showed rich diversity associated with *A. marina* rhizosphere. Nearly 21 prominent bands with 10–12 bands in each lane were eluted and sequenced. BLAST-N analysis of the *nosZ* gene and the protein-derived sequences showed 85–99% similarity and 83–98% similarities to unculturable *nosZ* gene, respectively. Nearly 18 sequences showed similarity to uncultured nitrous oxide reductase gene reported from various environmental sources while sequences of two

bands MSSRF Z10 and MSSRF Z18 were present in all the rhizosphere samples and showed 95–98% similarity to *Pseudomonas balearica* DSM 6083^T genome. Similarly, band MSSRF Z17 from *S. maritima* rhizosphere showed 99% similarity to *H. nitroreducens* LMG 24185^T nitrous oxide reductase gene which was also confirmed by nucleotide to protein translated sequences. which shared 75–100% identities to the *nosZ* sequences detected from a variety of marine environments including ocean sediments, sea sediments, salt marsh, freshwater, paddy soil, sewage water, solar saltern, laizhou bay soil, rhizosphere soil, *Puccinia distans* soil, agricultural and wheat soil. Phylogenetic analysis of protein-derived sequences showed six clusters forming a monophyletic clade with different known environmental sequences (Fig. S3 and Table S3). The results showed that the majority of the *nirS* and *nosZ* genes obtained through DGGE analysis belonged to the uncultured denitrifying bacterial group. Phylogenetic analysis of both *nirS* and *nosZ* genes formed clusters with ocean, marine, estuarine sediments, Landfill bioreactor, activated sludge and agricultural isolates which indicates the wide distribution of these genes, and yet to explore unknown bacterial lineages from this ecosystem (Figs. S2 and S3).

Diversity of culturable denitrifying bacteria

About 112 strains grew in nitrate broth, of which 83 strains were selected based on nitrate/nitrite reduction and identified as true denitrifiers using Greiss reagent (data not shown). All these strains were screened for nitrite reductase and nitrous oxide reductase genes as described in “Materials and methods”. Among the 83 *cdnirS* positive isolates only 74 isolates harbored *nosZ* gene with the amplicon size of 1100 bp compared to *Marinobacter hydrocarbonoclasticus* SP17^T and thus indicating the presence of both *nirS* and *nosZ* genes in 74 isolates while 9 isolates contained only the *nirS* gene. The genetic diversity among these 83 strains analyzed by BOX-PCR fingerprinting showed the presence of 24 clades at an 80% confidence level (Fig. 5a).

Taxonomy of denitrifying bacterial isolates

Nearly 96% of cultured denitrifiers isolated in this study belonged to the gammaproteobacterial, most of them were the well-known genus *Pseudomonas*. BLAST-N analysis (~ 1400 bp) of the 16S rDNA of nitrate-reducing bacterial groups revealed the predominant presence of genus *Nitratireductor aquimarinus* VL-SC21^T(2), *Staphylococcus hominis* DSM 20328^T(1), and *Bacillus aryabhattai* B8W22^T(2). BLAST-N analysis of denitrifiers was mostly represented by groups such as *Pseudomonas* sp. (48 isolates) comprising of *P. bauzanensis* DSM 22558^T, *P. xanthomarina* KMM1447^T, *P. baleriaca* DSM 6083^T, *P. stutzeri* ATCC 17588^T, and *P. xiamenensis* C10-2^T, *Paracoccus kondratievae* GB^Tsp. (4 isolates), *Labrenzia aggregata* IAM 12614^Tsp. (5 isolates), *Halomonas venusta* DSM 4743^T and *H. hydrothermalis* Slthf2^T (13 isolates), *Virgibacillus dokdonensis* DSW-10^T (4 isolates), and *Shewanella marisflavi* SW 117^T (3 isolates) (Fig. 5b).

Principal component analysis of DGGE fingerprints

Principal component analysis of all the three genes *nifH*, *nirS* and *nosZ* showed the qualitative differences in the distribution of genes among the rhizosphere regions. The PCA analysis separated the microbial communities into three different groups, well supported by UPGMA clustering analysis which represented the association of unique halophilic bacterial communities harboring these genes in halophytic rhizosphere compared to other mangrove rhizosphere. All the mangrove rhizosphere formed a unique clustering pattern as is revealed in PCA (Fig. S4a), Both the mangrove rhizospheres *A. marina* and *R. mucronata* exhibited almost similar gene distribution profiles and

formed a unique clustering pattern. Individual DGGE profile cluster analysis of these genes was well corroborated with the PCA analysis and UPGMA analysis (Fig. S4b).

Discussion

Bacteria are key mediators in maintaining the global biogeochemical cycling of essential elements like nitrogen, carbon, sulfur, and methane in the mangrove ecosystems. Studying microbial communities helps us to understand their essential role and contribute toward such phenomena and effective functioning of the ecosystem (Falkowski et al. 2008). Currently, the application of molecular techniques has been widely used for assessing the microbial diversity in various natural environments (Sousa et al. 2006; Muckian et al. 2007), A noticeable complication with the cultivation-based analysis is that only a miniature proportion of the microbial population can be retrieved from any sample, leaving the rest undetected or uncultured (Vartoukian et al. 2010).

In our study, the PCR-DGGE approach targeting the *nifH*, *nirS*, and *nosZ* genes from total soil DNA has been used to analyze the diversity of predominant unculturable nitrogen-fixing and denitrifying bacteria. The *nifH* genes have been used as marker genes for studying the nitrogen-fixing bacterial diversity and several bacterial groups harboring *nifH* genes have been reported in mangrove sediments, revealing high diazotrophic diversity in mangrove ecosystems (Zhang et al. 2008, 2017). Previous reports had suggested that nitrogen-fixing bacteria belonging to Proteobacteria were dominant in the marine ecosystem (Riemann et al. 2010), similarly in the present study also Proteobacteria predominantly harbored *nifH* genes contributing to nitrogen fixation in the mangrove ecosystem. In addition to proteobacterial groups, Firmicutes were also found to harbor *nifH* genes, Similarly, *nifH* gene sequences affiliated with alpha, beta, and Gammaproteobacteria, have been reported previously by Bagwell et al. (2002), from the tropical seabed grass which is in concurrence with our study. The findings of Bird et al. (2005) suggested that Gammaproteobacteria are predominant and acts as an important component of the heterotrophic nitrogen-fixing microbial community of the tropical and subtropical oceans. The sequence analysis of *nifH* DGGE showed similarity to uncultured nitrogen-fixing bacterial groups reported from high and low saline soils (Yousuf et al. 2014), marine sediments (Dang et al. 2013), rhizosphere of smooth cordgrass and salt marsh (Lovell et al. 2012), and agricultural soils (Pereira et al. 2013). None of the 29 *nifH* sequences reported in this study were related to earlier known *nifH* genes of cultured nitrogen-fixing bacteria reported neither from mangroves nor from

Fig. 5 a BOX-PCR Cluster analysis of culturable denitrifiers based on Dice coefficient and dendrogram construction by UPGMA algorithm. **b** Neighbor joining analysis of culturable denitrifiers based on 16S rDNA analysis. A bootstrap value of 1000 replicates have been carried out for analysis

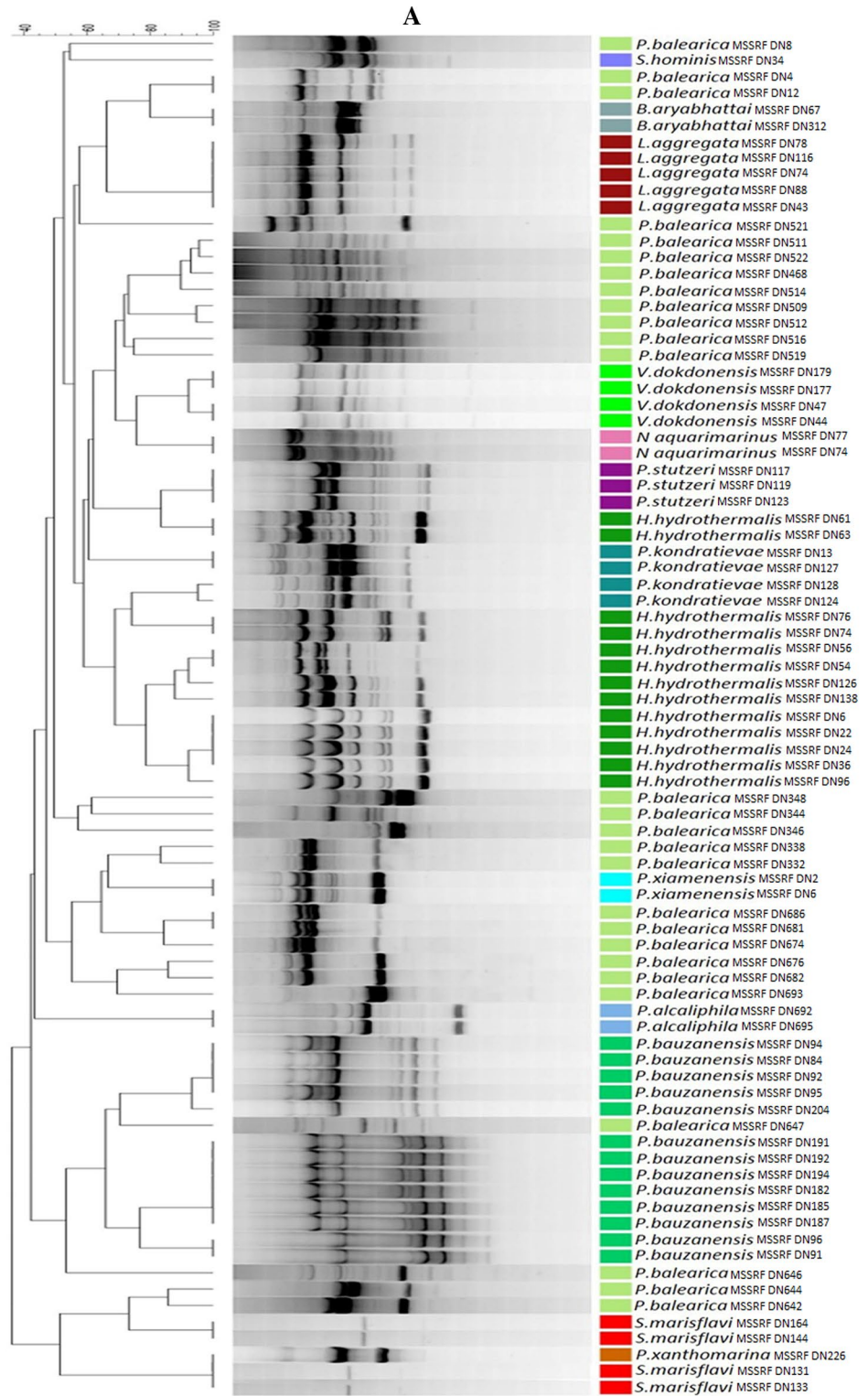
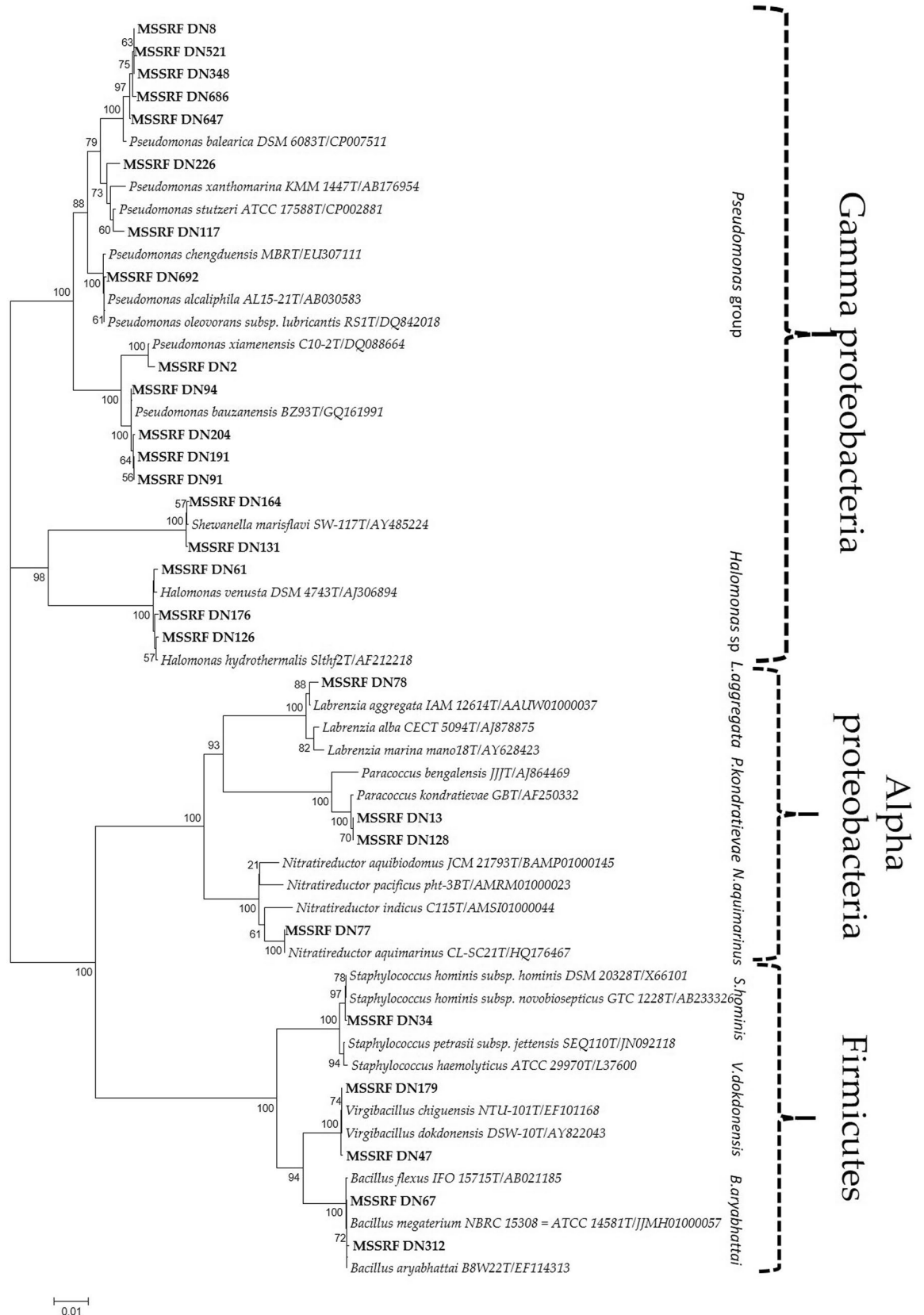


Fig. 5 (continued)

B



other ecosystems, indicating the abundance of unreported uncultured nitrogen-fixing bacteria in the mangrove rhizosphere soils. The phylogenetic placement of the *nifH* gene sequences from the mangroves exhibited unique *nifH* gene types affiliated with the phyla belonging to unculturable. The sequences were partially matching with the nitrogen fixers described in marine environments, and also those found in other ecosystems. Thus, the distribution of the *nifH* gene in the mangrove ecosystems represented both the marine and the terrestrial ecosystems.

Our current knowledge on the microbial community about the South Indian mangrove ecosystems is still largely based on cultivation-dependent studies (Rameshkumar et al. 2014; Viswanath et al. 2015; Raju et al. 2016). This needs to be further expanded using current molecular tools to completely understand the diversity of the nitrogen fixers associated with this ecosystem. The results obtained in our study showed that the majority of the culturable nitrogen fixers from the Pichavaram mangroves belonged to the Gammaproteobacterial group coinciding with earlier reports (Wu et al. 2016; Zhang et al. 2017; Liu et al. 2012; Rameshkumar et al. 2010, 2014; Viswanath et al. 2015). Flores-Mireles et al. (2007) showed that the nitrogen fixers isolated from the rhizosphere of mangroves were distributed to various genera such as *Azospirillum*, *Azotobacter*, *Rhizobium*, *Clostridium*, *Klebsiella*, *Vibrio*, *Phyllobacterium*, *Oceanimonas*, *Paracoccus*, *Corynebacterium*, *Arthrobacter*, *Aeromonas*, and *Pseudomonas*. In addition to these species, the present study also reported *Mangrovibacter* and *Rhodobacter* sp. The BOX-PCR profiling of vibrio consisting of *V. plantisponsor*, *V. alginolyticus*, and *V. neocaledonicus* (Fig. 2a) was supported by the phylogenetic analysis of these strains as they formed an outward clade with the type strains. From Ez-Taxon analysis, it is understood that the isolates of species *V. alginolyticus* and *V. neocaledonicus* cannot be distinguished based on 16S rDNA analysis, and intraspecies difference in BOX profile of these strains suggests that these strains may be novel species. On a similar note, so far only two *Mangrovibacter* species has been reported (Rameshkumar et al. 2010; Zhang et al. 2015) from the mangroves but the isolation of additional five *Mangrovibacter* species in this study displayed divergence from the reported strains in BOX-PCR profiling as well as phylogenetic analysis indicating they could be novel species. The genus *Bacillus* obtained in this study showed similarity to *B. aerophilus* 28K^T which has been previously reported in the stratosphere region of the earth's atmosphere by Shivaji et al. (2006). It is known that the strains *B. aerophilus*, *B. startosphericus*, and *B. altitudinis*, (Fig. 3a) cannot be differentiated by 16S rDNA analysis which is also well supported by BOX-PCR fingerprinting analysis. Our results confided the same thus suggesting that further experiments like multi locus sequence typing

(MLST) have to be done to prove that these might be novel species exhibiting diazotrophic activity.

Denitrification is well recognized as a dominant pathway for the removal of reactive nitrogen in marine sediments including polar sediments. A number of taxonomically unrelated bacterial groups belong to culturable and unculturable bacteria capable of carrying out denitrification in marine ecosystems have been reported (Arce et al. 2013). Several studies based on culture-independent approaches have been adopted to analyze the diversity of denitrifying genes like *narG*, *nirK*, *nirS*, *norB*, *norC*, and *nosZ* (Braker et al. 2000; Li et al. 2020; Gao et al. 2016) in forest and marine sediments. The *nosZ* gene, encoding N₂O reductase, an enzyme catalyzing the final step of denitrification, is largely unique to denitrifying bacteria. It represents the process leading to the loss of biologically available N from the sediments and has been used as a marker gene for determining the diversity of denitrifiers (Hong et al. 2019). The *nirS* (cd3afGC and R3Cd) and *nosZ* (nosZfGC and nosZ1773R) primer pair showed efficient amplification of the *nirS* and *nosZ* genes from the denitrifying populations of the four different mangrove rhizospheres. Studies by Lee et al. (2017) and Xie et al. (2020) showed the abundance of denitrifying bacterial communities in the pearl river estuary and San Francisco bay were correlated with the present study. As reported in other environmental studies of the functional genes in the denitrification pathway, most of the dominant *nirS* and *nosZ* types in our study clustered with other environmental clones. The majority of the sequences belong to uncultured denitrifying bacterial group reported from various environmental sources such as landfill leachate, estuarine sediments, activated sludge, salt marsh, forest soil (Bárta et al. 2010; Zheng et al. 2013) as well as sludge and agricultural ecosystem (Yoshida et al. 2012; Zhang et al. 2015), suggesting that mangroves harbor denitrifying bacterial communities from both tidal and urban ecosystems.

The exploration of the culturable diversity of these *nirS* and *nosZ* in culturable heterotrophic bacterial isolates indicated the prominent distribution of these genes in the Gammaproteobacteria group (Qaisrani et al. 2019). The results obtained were on par with the previous studies on marine sediments (Bowman et al. 2005), which revealed that Gammaproteobacteria was the most abundant denitrifying population in mangroves. Studies by Fernandes et al. (2012) also showed the dominance of Gammaproteobacteria in culturable and non-culturable denitrifiers from Tuvem and Divar estuary. Our results also were concurrent to earlier reports with predominant denitrifying communities belonging to Gammaproteobacteria consisting of *Pseudomonas* and *Halomonas* groups as predominant denitrifiers (Bowman et al. 2005; Fernandes et al. 2012).

In this study, we were able to successfully screen and characterize some of the aerobic culturable heterotrophic denitrifying bacterial populations from this ecosystem. In the culturable analysis of denitrifiers, it was observed that the majority of the isolates were from the gammaproteobacterial group which belonged to the genus *Pseudomonas* sp. A different group of bacterial genera like *Halomonas*, *Labrenzia*, *Paracoccus*, *Nitratireductor*, *Bacillus*, *Virgibacillus*, *Shewanella*, *Staphylococcus* Was also observed to contribute to the denitrifying activity. Previous known reports have shown that these microbial groups have been described from different ecosystems i.e., *Halomonas* from the hydrothermal vent (Kaye et al. 2011), *Labrenzia* from the marine ecosystem as well as from halophytic plant *Sueada* (Bibi et al. 2014) which were similar to the findings in this study. Other groups like *Paracoccus* (Flores mirles et al. 2007), *Nitratireductor* (Labbe et al. 2004), and *Virgibacillus* (Yoshida et al. 2012) were reported from marine as well mangrove ecosystems except for *Bacillus* which has been reported from the stratosphere (Shivaji et al. 2006). It was observed that the genus *Pseudomonas*, *Labrenzia*, *Halomonas*, *Paracoccus*, *Virgibacillus*, and *Shewanella* were found to harbor both *nirS* and *nosZ* gene whereas other genera like *Bacillus*, *Staphylococcus*, and *Nitratireductor* harbored only the *nirS* gene. The denitrifying *Pseudomonas* comprised diversified species such as *P. balearica*, *P. bauzanensis*, *P. xiamanensis*, *P. stutzeri*, and *P. xanthomarina*. This is the first study to describe the presence of *P. balearica*, *P. bauzanensis*, *Labrenzia* sp. and *Paracoccus kondratievae* from the mangrove ecosystem and were found to be vigorous denitrifiers as they can convert nitrate into the gaseous form of nitrogen within 24 h of incubation under aerobic conditions.

A strong correlation between the DGGE profiles of denitrifiers and culturable denitrifiers was observed. Some of the sequences of *nirS* showed similarity to *P. balearica* and *nosZ* gene to *Halomonas nitroreducens* which has been observed in culture-dependent studies as well. The study revealed that 80% of the denitrifiers belonged to *Pseudomonas* sp. and *Halomonas* sp. represented 16% indicating the dominance of these two species in the rhizosphere contributing to denitrification. Overall, the results obtained in this study corroborated with the previous studies of marine sediments which showed Gammaproteobacteria as the most abundant nitrogen-fixing and denitrifying population.

Conclusion

This is a basic study in an attempt to explore the diversity of culturable and unculturable microbial groups involved in the nitrogen fixation and denitrification process. To our knowledge, this is the first paper attempting to explore the microbial communities involved in the nitrogen fixation

and denitrification process through culture-dependent and independent analyses from Pichavaram mangroves. The results presented here provide baseline data about nitrogen-fixing and denitrifying bacterial groups present in mangrove rhizosphere regions at the genetic level. It is essential to mention that two strains from the Rhodobacteraceae family namely *Rhodobacter johrii* and *Labrenzia aggregata* were involved in the nitrogen fixation and denitrification process; sulfur cycling bacteria *Catenococcus thiocycli* involved in nitrogen fixation and nitrate-reducing bacteria *Nitratireductor* are being reported for the first time from the mangrove ecosystem. Few novel groups belonging to *Vibrio*, *Mangrovibacter*, *Catenococcus*, and *Bacillus* were identified based on BOX-PCR fingerprinting analysis and need further analysis. DGGE analysis revealed the presence of many uncultured bacterial groups harboring genes involved in the nitrogen fixation and denitrification process. Overall, this is the first study in an attempt to under the microbial diversity where DNA samples were pooled and analyzed using PCR-DGGE technique in combination with culture-dependent studies which revealed known as well as unknown microbial groups involved in the nitrogen cycling process in this study. Though there is a limitation in the present study with respect to replicates used, the findings indicate the microbial richness associated with the Pichavaram mangrove rhizospheres. Further research has to be carried out to understand the expression of these genes under different conditions which will give complete data on the microbial communities involved in this process.

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Author contributions BV: experiment designing, data analysis, and writing. VRP: supervision and critical correction of the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals The study is not related to animals or humans.

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