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Occurrence of two endophytic associative nitrogen-fixing *Caulobacter* spp., from three non-nodulating endemic legumes based on *nif*H gene analysis

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ABSTRACT

Legumes are cosmopolitan plants, and nodulating legumes are well known for their symbiotic nitrogen-fixing ability by rhizobia-legume interaction. Biological nitrogen fixation in non-nodulating endemic legumes by associative or endophytic symbiotic bacteria requires much needed attention. Three non-nodulating legumes, namely, *Humboldtia brunonis* Wall., *Kunstleria keralensis* C.N. Mohanan and N.C. Nair, and *Bauhinia phoenicea* Wight and Arn., endemic to the Western Ghats regions of Karnataka state, were studied. Employed techniques of selective culture media to understand diazotrophic diversity inside the roots of these plants. The isolates that can grow in a nitrogen-free semisolid agar medium have been considered positive for nitrogen-fixing ability. nifH gene is taken as the marker gene to ascertain the nitrogen-fixing ability of the bacteria. The qualified bacterium in the previous steps is identified using 16s RNA sequencing and the Sanger sequence method. The results obtained showed *B. phoenicea* Wight and Arn. and *H. brunonis* Wall. as *Caulobacter segnis*, and in *K. keralensis* C.N. Mohanan & N.C. Nair, it is *Caulobacter crescentus*. The presence of the nifH gene is demonstrated through molecular methods. This work adds to the diverse works of *Caulobacter* as a successful plant growth-promoting endophyte even in the nitrogen-deficient, slopy soils of the Western Ghats.

1. INTRODUCTION

The nitrogen chemistry makes it a limiting nutrient for plants [1]. Nitrate, nitrite, or ammonia forms of nitrogen are convenient for plant uptake and assimilation [2,3]. Biological nitrogen fixation, essentially a prokaryotic ability, converts the atmospheric nitrogen (N_2) into the plant assimilable forms [4]. The bacteria depend on less oxygenic areas like the root endosphere to perform this fixation. The free-living rhizospheric bacteria thus enter inside the plant's root and perform the activity with the help of its molecular machinery [5].

The Western Ghats contain 756 Leguminosae members, of which 58 are endemic to the region, and 14 are reported from Karnataka state [6]. These legumes' life forms are trees and lianas. Among these endemic plants, *Humboldtia brunonis* Wall., *Bauhinia phoenicea* Wight and Arn., and *Kunstleria keralensis* C.N. Mohanan and N.C. Nair were considered in this study. They thrive in these tropical rainforest areas experiencing heavy rains up to 5000 mm during southwest monsoon months and the surplus water leading to nitrogenous nutrients leaching. Neutral pH due to the rainfall and soil erosion and the high rate of runoff from the slopy regions add to the problem. During non-monsoon months, the region's temperature rises, influencing water draining from its reservoirs in the forest. Soil parameters, including pH, temperature, salinity, and decomposition rates, also shift to another extreme [7]. All these events cumulatively put abiotic and biotic stress on these endemics. Similar stress will be experienced by countless microorganisms living in this area. This abiotic stress has induced evolutionary adaptation strategies and many plant-microbe associations to mitigate the conditions with mutual benefits.

Beneficial bacteria are sheltered within the rhizosphere, rhizoplane, and endosphere zone to overcome nitrogen scarcity. In the present study, the isolation of nitrogen-fixing microbes from the endosphere zone using NFb media [8] gave the culturable bacteria from all the three selected plants. The ability to fix nitrogen by these bacteria was analyzed using IGK3/DVV primers as they can express the *nif*H gene in polymerase chain reactions [9,10].

2. MATERIALS AND METHODS

2.1. Collection of Roots

The feeder roots of *H. brunonis* Wall. [11], *B. phoenicea* Wight and Arn. [12], and *K. keralensis* C.N. Mohanan and N.C. Nair [13] were collected from their natural habitat (12°43'47.4"N 75°39'40.0"E) in the Western Ghats of Karnataka state. Care has been taken to minimize

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the damage to the lateral root as these plants are endemic, rare, and critically endangered [14].

2.2. Surface Sterilization of Roots

The feeder roots were cleaned with running tap water to yank dirt particles. Then, the roots were immersed in a 2% sodium hypochlorite solution for 2 min. Repeatedly rinsed with sterile distilled water, then sliced into 1 cm long parts using a sterile blade and stored in sterile distilled water for further use [15].

2.3. Preparation of New Fabio (NFb) Medium

The NFb medium contains (g L^{-1}): Malic acid – 5.0; K_2HPO_4 – 0.5; $MgSO_4.7H_2O$ – 0.2; NaCl – 0.1; $CaCl_2.2H_2O$ – 0.02; $CuSO_4.5H_2O$ – 8×10^{-5}; $ZnSO_4.7H_2O$ – 2.4×10^{-4}; H_3BO_3 – 2.8×10^{-3}; $Na_2MoO_4.2H_2O$ – 2×10^{-3}; $MnSO_4.H_2O$ – 2.35×10^{-3}; bromothymol blue 0.01; FeEDTA 6.56×10^{-2}; Biotin-1×10^{-2}; Pyridoxal-HCl-2×10^{-2}; KOH-4.5 g; distilled water to bring the final volume to 1000 mL; and pH is adjusted to 6.5. A quantity of 1.80 g agar L^{-1} is added to prepare the semisolid medium and 15 g agar L^{-1} for the solid medium. Due to the high pH, the various ingredients were added in the given sequence to avoid precipitation of iron or other salts [8].

2.4. Growing the Bacteria in Selective Media

Test tubes fitted with a cap are filled with about 5 mL NFb media to obtain culture. Root segments are stubbed into the tubes aseptically. These tubes are incubated at room temperature for about 2–3 days. A pellicle formation at the sub-surface level of the media is a positive indicator of the growth of diazotrophic bacteria. This pellicle formed is subcultured to fresh media to ensure reappearance of the pellicle. After successfully growing the pellicle in two subcultures, later, the pellicle is transferred to solid NFb media with subculturing based on need. From this method, pure colony of diazotrophic bacteria obtained and used further for molecular characterization and identification processes. All these cultures are maintained in triplicates.

2.5. Identification of Bacteria

DNA isolation was carried out with the CTAB method. The cultures grown on solid NFb media are harvested using centrifugation in the form of pellets and collected. To the pellet, added 500 µL of CTAB extraction buffer and mixed with a vortex mixer. The homogenate mixture transferred to a 60°C bath for 30 min. Following the incubation period, centrifugation of the homogenate for 5 min at 14,000 × g was carried out. Added an equal volume of chloroform/isoamyl alcohol (24:1) mixture. Vortexed for 5 s, then centrifuged the sample for 5 min at 14,000 × g to separate the phases. The aqueous upper phase is transferred to a new tube. Precipitated the DNA by adding 0.7 volume cold isopropanol and incubated at −20°C for 30 min. Sample subjected to centrifugation at 14,000 × g for 10 min. Decanted the supernatant without disturbing the pellet and subsequently washed with 500 µL ice-cold 70% ethanol. Ethanol is decanted. Removed the residual ethanol by drying in a SpeedVac. The pellet was dried long enough to remove alcohol without completely drying the DNA. Obtained DNA was dissolved in 30 µL TE buffer. The pellet was warmed to dissolve. Added 1 µl of RNase solution A and incubated at 37°C for 30 min. The DNA thus obtained is quantified [16]. The obtained DNA is purified by column purification method of sample (Kit method), where added 200 µL of binding buffer to DNA and mixed well, transferred to the column (750 µL each time), spun at 12,000 × g for 1 min, added wash buffer 750 µL, and spun at 12,000 × g for 1 min. Repeated the wash buffer step, dry spun for 2 min, added 20 µl of elution buffer, and spun at 12000 × g for 1 min. The obtained DNA is checked for its purity at 260 nm for absorbance of 1.0. This high-quality DNA is used for 16S rRNA gene-based bacterial identification.

2.6. Randomly Amplified Polymorphic DNA

RAPD was performed for genomic DNA from the bacterial samples [17]. Amplified 16S rRNA gene to check genetic variation among obtained genotypes using the following PCR conditions: Initial denaturation 95°C for 2 min, final denaturation 95°C for 30 s, annealing 50°C for 30 s elongation 72°C for 1 min, repeated steps 2, 3, and 4 for 30 cycles, final elongation 72°C for 10 min with a hold at 4°C forever using 27F (5'-AGAGTTTGATCCTGGCTCAG-3' with 53°C annealing temperature) and 1492R(5'-GGTTACCTTGTTACGACTT-3' with 57°C annealing temperature) primers [18]. Each PCR reaction for testing the amplification efficiency and development of multiplex PCR assays for DNA barcode primers contained 1 µL DNA template (25 ng), 2 µL 10× reaction buffer, 0.5 µL $MgCl_2$ (50 pM), 1 µL dNTPs mix (10 mM), 1 µL forward primer – 27F (10 pM), 1 µL reverse primer – 1492R (10 pM), 0.5 µL Taq polymerase (5 U/pi), and the final volume 25 µL adjusted with molecular grade water.

2.7. Identification of Isolates by 16S rRNA Sequence Analysis

Genomic DNA from the bacterial samples was taken, and 16srRNA was amplified with the procedure explained above in section 2.5. Agarose gel eluted PCR products of the 16S rRNA gene are purified and sequenced. Gel purification protocol by cutting the required DNA band on the gel, adding 600 µL of gel solubilization buffer, and heating it at 55°C until the gel dissolves completely, to this added 200 µL of isopropanol with proper mix. This gel is transferred to the column, spun at 12,000 × g for 1 min, later added 700 µL of wash buffer, spun at 12,000 × g for 1 min, subjected to dry spin for 2 min, added 20 µL of elution buffer, and spun at 12,000 × g for 1 min. This step is followed by subjecting it to Sanger sequencing PCR with the conditions of initial denaturation at 95°C for 2 min, final denaturation at 95°C for 30 s, annealing at 50°C for 30 s, termination at 60°C for 4 min, repeated the steps 2,3 and 4 for 30 cycles, and hold at 4°C forever. Post-sequencing and PCR purification are performed by adding 125 Mm 2.5 µL EDTA to each well and giving a short spin. Then, added 35 µL of ethanol using a multichannel pipette, which is subjected to vertexing for 10 min at 2000 × g, later centrifuged at 3510 × g for 30 min; using tissue bed, decanted ethanol at 300 × g (by inverting the plate for 30 s), added 40 µL of 80% ethanol to the wells, and centrifuged at 3510 × g for 12 min. On completion, air-dried for 30–45 min, covering the plate with lint-free tissue; added 13 µL of HiDi Formamide and gave a 1 min spin, denatured at 95°C for 5 min, and placed the plate in the sequencer.

2.8. *Nif*H Gene Analysis

Genomic DNA is taken to analyze the *nif*H gene. For this study, PCR conditions are initial denaturation of 95°C for 2 min, final denaturation of 95°C for 30 s, annealing 50°C for 30 s, elongation of 72°C for 1 min, repeated the steps 2, 3, and 4 for 30 cycles, final elongation at 72°C for 10 min, and hold at 4°C forever. IGK3 (GCIWTHTAYGGIAARGGIGGIATHGGIAA) is the forward primer, and DVV (ATIGCRAAICCICCRCAIACIACRTC) is the reverse primer used for this purpose [19,20]. Agarose gel eluted PCR products of the *nif*H gene are purified and sequenced [21].

2.9. Data Analysis

Genomic DNA isolated from samples was analyzed for its purity. The agarose gel electrophoresis is compared with the 1 kb ladder. Binary matrix generated from banding pattern for the RAPD study. Sequencing files obtained in.AB1 format are viewed using FinchTV, BioEdit, Chromas Lite, and SeqScanner software. The quality of the obtained sequence is observed through electropherogram peaks. Analyzed the sequencing data using BLAST servers [22]. The obtained results were used to construct phylogenetic trees using Mega-X [23]. nifH gene database (nifH_database_2012.fasta.) is used to obtain relevant sequences for analysis [24].

3. RESULTS AND DISCUSSION

The feeder roots of these plants yielded pellicles of bacterial growth in the NFb semisolid medium, indicating the isolation of nitrogen-fixing organisms endophytic to the respective plant roots. The pellicle formed was subjected to RAPD and 16SrRNA gene analysis to identify the residing organism [Figure 1], and the results showed the presence of two species of *Caulobacter*. In *B. phoenicea* Wight and Arn. and *H. brunonis* Wall., endophytic bacterium is identified as *Caulobacter segnis* [Figure 2], and in *K. keralensis* C.N. Mohanan and N.C. Nair, the obtained endophyte is identified as *Caulobacter crescentus* [Figures 3 and 4]. Analyzed these bacteria further for the *nif*H gene using alphaproteobacteria-specific primers for *the nif*H gene, namely, IGK3/DVV, to amplify the gene in PCR. Phylogenetic trees are compared with the nifH gene database and BLAST server sequences for the *nif*H gene, which shows a distinct association with already reported diazotrophic bacteria [Figure 5].

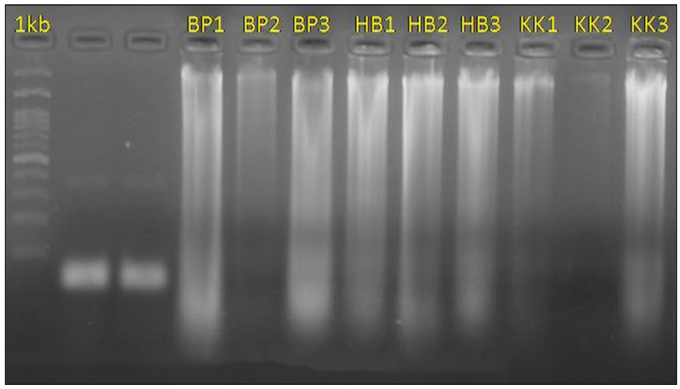

Figure 1: Genomic DNA isolated from samples and compared with 1 kb ladder.

H. brunonis Wall., *K. keralensis* C.N. Mohanan and N.C. Nair, *and B. phoenicea* Wight and Arn. are the three endemic legumes studied for the possible diazotrophic endophytes. Among the selected legumes, taxonomically, *B. phoenicea Wight* and *Arn.* belongs to the subfamily Cercidoideae, *H. brunonis Wall.* belongs to Detarioideae, and *K. keralensis C.N. Mohanan* and *N.C. Nair* belongs to Papilionoideae [6]. These three subfamilies are phylogenetically shown the varied nature of plant-microbe interactions. Their conservation status is also under the rare, endangered, and endemic category [14,21]. Since these plants shared a similar microclimate in the study area, the specific association with *Caulobacter* spp. has emerged as a potential plant growth-promoting endophytic association for these three endemics. *Caulobacter* is also a beneficial endophyte in many plants like rice [25,26] and sugarcane [27]. The present study determined the *nif*H gene sequence from these bacteria. Nitrogen assimilation by the microorganisms in all three plants is mediated by *Caulobacter* spp., which may have specific evolutionary significance. Berrios and Ely, 2020, opinion that plant growth enhancement is not a conserved feature in the Caulobacter genus [28]. They have not come across nitrogen fixation ability by studying *the nif*A, but in this study, specific primers on the nifH gene have determined its presence. The ability to fix atmospheric nitrogen in these nitrogen-deficient soils could have partially led to these plants' endemism as this association is not as strong as rhizobia-legume. As all the three plants show perennial life forms, the plants' strategy to adopt the dynamics of their microclimate may support *Caulobacter* spp. to thrive as a successful endophyte. The *nif*H gene of *Caulobacter* indicates its diazotrophic activity confirming it as plant growth-promoting endophytic bacteria.

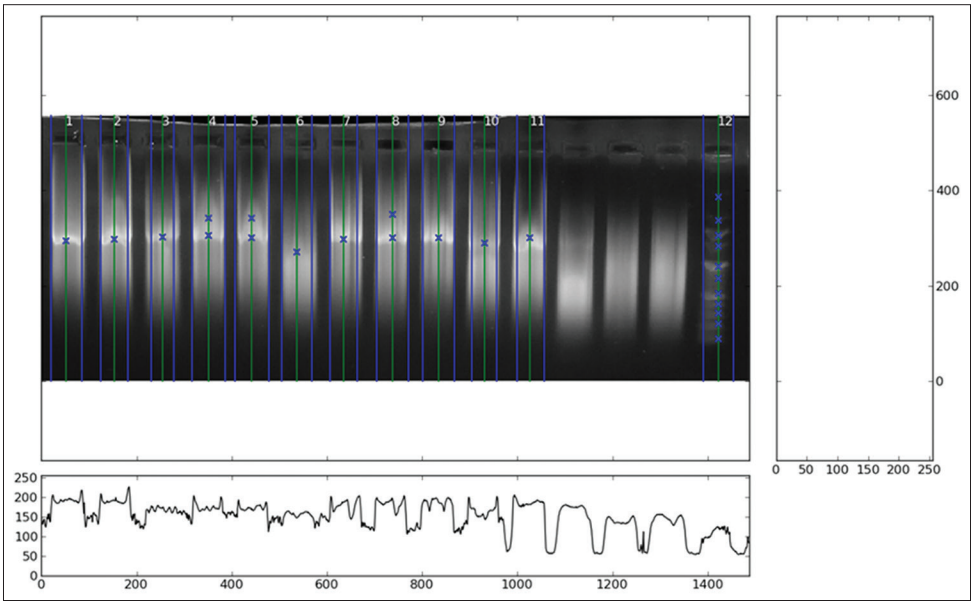

Figure 2: 16srRNA bands of all genotypes compared with 1 kb ladder.

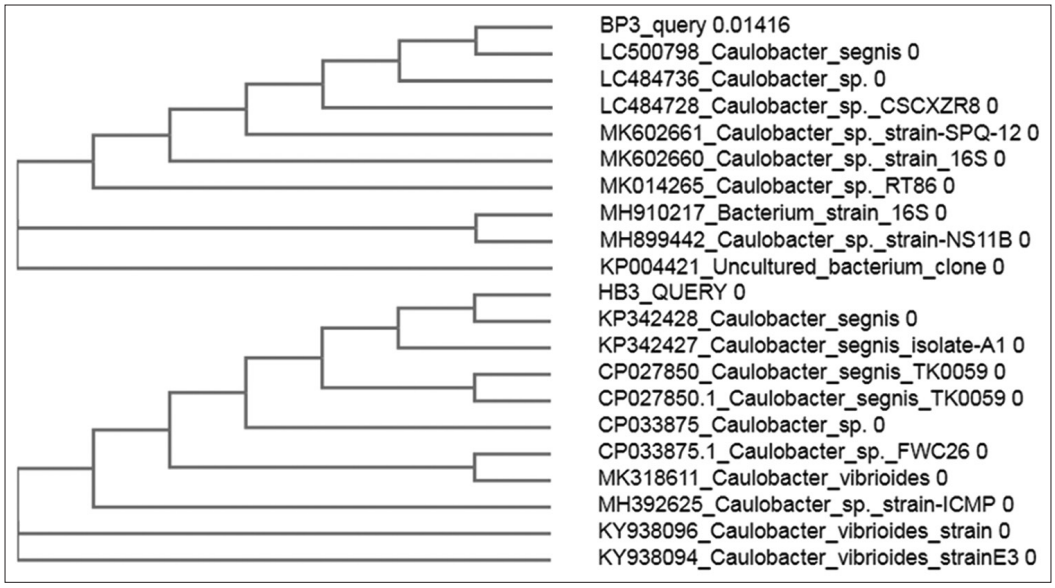

Figure 3: Phylogenetic tree obtained for *Bauhinia phoenicea* weight and Arn. and *Humboldtia brunonis* Wall. endophyte *Caulobacter segnis.*

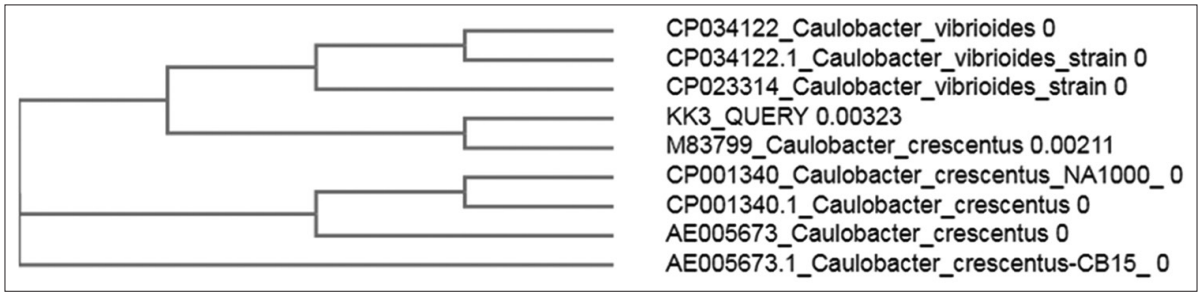

Figure 4: Phylogenetic tree obtained for *Kunstleria keralensis* C.N. Mohanan and N.C. Nair endophyte *Caulobacter crescentus.*

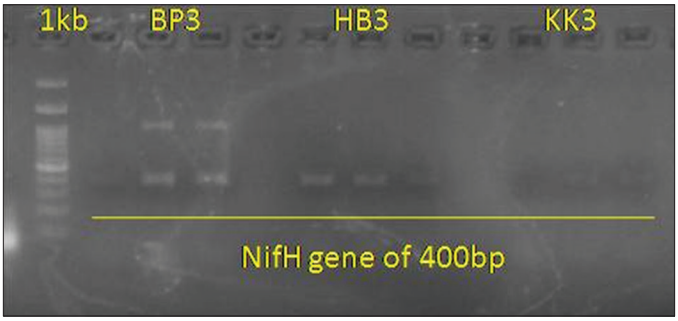

Figure 5: Agarose gel electrophoresis showing the presence of *nif*H gene isolated from the endophytes of three legumes.

4. CONCLUSIONS

The present study of endophytic bacteria residing in Western Ghats' three legumes has shown positive results for *nif*H gene analysis. This way of nitrogen fixation may be one of the parallel strategies in nitrogen fixation adopted by these non-nodulating legumes. This report also indicates the further analysis of the growth promotion ability of these species. Further study on the plants of this locale may explore the usefulness of this bacterium in this local agriculture. Further characterization of these strains with their plant growth-promoting potential may serve a significant role in the conservation efforts of these endemics.

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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