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Influence of genotype, nodule position, and edaphic factors on microbial diversity and assembly of pigeonpea (*Cajanus cajan*) root nodules in Indian soils

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Abstract

Background Pigeonpea (*Cajanus cajan*) is an important legume crop in semi-arid regions with multiple uses. The microbial diversity within its root nodules in Indian soils remains poorly explored. We investigated the bacterial diversity of pigeonpea root nodules across different genotypes and soil types to identify the factors driving their assembly. Using a metagenomic approach and high-throughput sequencing of the 16S rRNA gene, we analyzed the nodule microbiomes of three pigeonpea genotypes (Asha, Durga, and Mannem Konda Kandi) grown in three different soil types (Alfisol, Vertisol, and Inceptisol) and wild pigeonpea (*C. scarabaeoides*) in its native soil.

Results Our results indicated that pigeonpea nodules harbor diverse rhizobial and non-rhizobial endophytes and that host genotype, nodule position, soil type, and other edaphic factors influence significant variation in the microbial community structure. The core nodule microbiome was dominated by Proteobacteria and Bacteroidetes. *Bradyrhizobium* and *Ensifer* were predominant among the rhizobial taxa, and non-rhizobial genera such as *Pseudomonas*, *Chitinophaga*, and *Limnobacter* were also abundant. Edaphic factors, particularly soil type, pH, and nutrient availability, had a stronger influence on the nodule bacterial community composition than the host genotype. Although bulk soil exhibited higher bacterial diversity, nodule microbiomes were less diverse but more specialized, indicating host-mediated selection. A comparison of the nodule microbiomes of wild and cultivated pigeonpea revealed distinct differences, with the core nodule microbiome of wild pigeonpea dominated by *Bradyrhizobium*, while that of cultivated pigeonpea exhibited a diverse bacterial community.

Conclusions These findings demonstrate that soil properties play a more critical role than host genetics in shaping the pigeonpea nodule microbiome, emphasizing the importance of environmental conditions in symbiotic interactions. The differences between wild and cultivated genotypes suggest that domestication has altered

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microbial recruitment strategies. This study provides foundational insights into the factors driving microbial assembly in pigeonpea nodules, with implications for improving crop productivity through targeted microbial management. Future research should explore the functional roles of these microbial communities to optimize their use in sustainable agriculture.

Keywords Edaphic factors, Genotype influence, Nodule microbiome, Non-rhizobial endophytes, Pigeonpea, Rhizobia, Soil type

Background

Pigeonpea [*Cajanus cajan* (L.) Millspaugh], a vital legume food crop, has diverse applications as food, feed, fodder, and fuel. Like other legumes, it also enriches soil through biological nitrogen fixation. Globally, pigeonpea cultivation spans approximately 6.0 million hectares [1], primarily as a rain-fed crop in the semi-arid tropical and subtropical regions of South Asia, East Africa, Latin America, and the Caribbean [2, 3]. Over a billion people in developing countries rely on pigeonpea as their primary dietary protein source. Millions of resource-poor smallholder farmers cultivate this multipurpose crop to sustain their livelihoods, often with limited inputs. Domestication of its wild progenitor species, *Cajanus cajanifolius* (endemic to the Indian subcontinent), gave rise to cultivated pigeonpea in central India more than 3,500 years ago, subsequently leading to its global expansion [2, 3].

India is the world's leading producer of pigeonpea, accounting for 72% of the global supply [1]. Among cultivated legumes in India, it ranks second after chickpea, contributing 15% of the area under cultivation and 17% of the total pulse production [4]. The major pigeonpea growing zones in India can be divided into three distinct regions: the southern zone (comprising Andhra Pradesh, Karnataka, and Telangana), the central zone (encompassing Madhya Pradesh, Gujarat, and Maharashtra), and the northern plain zone (primarily Uttar Pradesh) [5]. Notably, pigeonpea yields in Andhra Pradesh, Madhya Pradesh, and Uttar Pradesh consistently surpass the national average [4]. The predominant soil types in these states are red soil (Alfisol) in Andhra Pradesh, black soil (Vertisol) in Madhya Pradesh, and alluvial soil (Inceptisol) in Uttar Pradesh [6].

Rhizobia were once considered to be the sole inhabitants of legume nodules. Several rhizobia can colonize nodules of the same plant and can even co-occupy the same nodule [7, 8]. Recent culture-dependent and culture-independent approaches have confirmed the existence of a diverse nodule microbiome, suggesting that rhizobia coexist with non-rhizobial nodule endophytes [9, 10], challenging the widely accepted notion that individual nodules typically harbor only one rhizobial strain. Instead, nodules may harbor multiple species, including non-rhizobial endophytes [11], also known as 'non-rhizobial root nodule endophytes' (NREs) [12].

Pigeonpea can establish symbiotic associations with diverse rhizobial genera [13]. Culture-dependent studies suggest that pigeonpea can be nodulated by *Bradyrhizobium* spp. [14], *Ensifer* (formerly *Sinorhizobium*) spp. [15, 16], *Rhizobium* spp. [17, 18], *Mesorhizobium* spp. [15, 17], or even *Burkholderia* (reclassified as *Paraburkholderia*) spp. [17], indicating that pigeonpea may be promiscuous in recruiting rhizobia for nodulation in Indian soils. However, in certain geographical regions such as the Dominican Republic [19, 20], Côte d'Ivoire [21], Brazil [22], and Ethiopia [23], pigeonpea plants form nodules exclusively with *Bradyrhizobium* spp. or *Ensifer* spp. [13, 24], suggesting that additional species reported in nodulation studies may require re-evaluation. In addition to rhizobial symbionts, pigeonpea roots harbor a diverse community of non-rhizobial colonizers, including bacteria from the genera *Agrobacterium*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Brevibacillus*, *Chryseobacterium*, *Enterobacter*, *Klebsiella*, *Lactobacillus*, *Paenibacillus*, *Pseudomonas*, *Serratia*, *Streptomyces*, and others [21, 25, 26].

Whole-genome sequencing has enabled the characterization of the genetic and genomic diversity of pigeonpea, which has a genome size of 833.07 Mb [3, 27, 28], while the nodule microbiome remains largely uncharacterized. The nodule microbiomes of legumes such as common bean [29, 30], *Medicago truncatula* [31], clover [32], soybean [33], lentil [34], groundnut [35], *Sophora davidii* [36], *Alnus* spp. [37, 38], *Prosopis cineraria* [39], *Desmodium* spp. [40], chickpea [41], and sea buckthorn [42] have been reported in different countries but poorly explored in India. A comprehensive study of the nodule-associated bacterial community in Indian soils or high-throughput screening of common symbionts of pigeonpea is required for a crop that has evolutionary roots in India, the largest pigeonpea-producing nation. The present study analyzed the microbial diversity of pigeonpea root nodules in major Indian soils and evaluated the factors driving nodule microbiome assembly.

We assessed the bacterial diversity associated with pigeonpea in Indian soils to identify whether there is a core nodule microbiome. In addition, the influences of edaphic factors, geoclimatic conditions, host characteristics, and agricultural practices on the nodule microbiota and its spatial distribution across different pigeonpea genotypes were examined. We also compared the nodule

microbiomes among multiple pigeonpea genotypes (considering three genotypes and three cultivated soil types) and their wild relative *Cajanus scarabaeoides* from their native soils. We hypothesized that the composition and diversity of the nodule bacterial community, especially the relative abundance of rhizobial and non-rhizobial taxa, may vary between the different genotypes and soil types and be influenced by environmental and host factors. Further, we presumed wild pigeonpea to host a distinct nodule microbiome due to its adaptation to native soil environments.

Methods

Seed material

Three popular pigeonpea cultivars (genotypes) with unique physiological and agronomical traits *viz.* Mannem Konda Kandi (MKK; ICPH-2740), Asha (ICPL-87119), and Durga (ICPL-84031), were selected for this study. The seeds were procured from the International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India. Before sowing, seeds were surface-sterilized using 0.1% mercuric chloride ($HgCl_2$) and 70% ethanol, then germinated on Murashige and Skoog agar medium.

Soil sampling

Soil samples were collected from pigeonpea fields across multiple locations to assess the impact of soil type and genotype on the pigeonpea nodule microbiome. Three distinct soil types—Alfisols (Rompicharla, Andhra Pradesh; 16.213900 N, 79.921386 E), Vertisols (Athner, Madhya Pradesh; 21.6406552 N, 77.91300 E), and Inceptisols (Sitamarhi, Uttar Pradesh; 25.2782289 N, 82.28691 E)—were sampled from farmers' fields during the pre-sowing season in June 2017. Additional soil samples were collected from 92 locations across major pigeonpea-growing states in India (Andhra Pradesh, Telangana, Madhya Pradesh, and Uttar Pradesh). These regions include diverse soil types, geoclimatic conditions, and agricultural practices (e.g., cropping patterns, irrigation, and fertigation). Physicochemical characterization of all soils was performed at the Charles Renard Analytical Laboratory, ICRISAT, using standard analytical methods (Additional File 1).

Pigeonpea nodule microbiome in multiple genotypes

Three pigeonpea genotypes (Asha, Durga, and MKK) were grown in three different soil types (Alfisols, Vertisols, and Inceptisols) to assess the relative contributions of soil type and genotype to the nodule microbiome. Three plants of each genotype were transplanted into pots (7.5 kg capacity) filled with respective soil types. The plants were grown in six biological replicates in a glasshouse under identical light, temperature, and humidity conditions until the peak vegetative stage (four weeks). Six pots of soil for each soil type (without growing any plants) were used as bulk soil controls. The plants and control pots were watered as required with sterilized distilled water every other day without any further fertilization. Nodules were harvested at the vegetative stage (28 days after seedling emergence) and stored at -80 °C until DNA isolation.

Pigeonpea nodule microbiome in multiple soil types

A single pigeonpea genotype (MKK) was grown in 92 different soil types to study the influence of agroclimatic conditions on nodule microbiomes. The MKK genotype (ICPH-2740) was chosen based on its widespread cultivation and well-documented agronomic performance in diverse agroclimatic conditions. Plants were grown in three biological replicates in different soils in a glasshouse under identical light, temperature, and humidity conditions for up to 28 days until the plants formed mature nodules. The plants and bulk soil control (pre-sowing soils without plants) pots were supplied with sterilized distilled water, as needed, without fertilizer. The plants were harvested during the mature nodule formation stage (28th day). Nodules from three biological replicates were pooled together, and four bulk soil samples (control) were preserved at -80 °C until processing.

Spatial distribution of the nodule microbiome

To study the spatial distribution of nodule microbiomes and assess whether nodule microbiome composition varied with nodule position and host genotype, three pigeonpea genotypes (MKK, Asha, and Durga) were grown in a single soil type (red soil) in a glasshouse under identical conditions of light, temperature, and humidity for up to 28 days. Individual nodules were carefully harvested and stored at -80 °C until metagenomic DNA isolation. Nodules were classified into three groups based on their position on the root system: primary roots (taproots), secondary roots, and tertiary roots (Supplementary Fig. S1, Additional File 2). Three root nodules per plant were randomly selected across the entire root system, with one nodule each from primary, secondary, and tertiary roots. The microbiomes of 27 individual pigeonpea root nodules from nine plants (three plants of each genotype) and three bulk soil (pre-sowing) samples were analyzed via high-throughput DNA sequencing.

Wild pigeonpea (*C. scarabaeoides*) nodule microbiome

Wild pigeonpea (*C. scarabaeoides*) plants were sampled in situ from their natural habitat at the University of Hyderabad campus (17.457462 N, 78.314313 E) in Rangareddy district, Telangana, India, during the peak vegetative stage (60 days after seedling emergence). University of Hyderabad has a tropical environment with a

dry deciduous biome and scrubby vegetation, with an average annual temperature of 24 °C (16.1–38.3 °C) and an average annual precipitation (rainfall) of 956.55 mm. Nodules were harvested manually from the wild pigeonpea roots and processed for DNA isolation.

Metagenomic DNA extraction, PCR, and sequencing

Metagenomic DNA was extracted from the bulk soil and nodules (0.3–0.5 g for each) using a NucleoSpin® Soil Kit (Machery Nagel, Germany) according to the manufacturer's instructions. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the 515F/806R primer pair [43]. The PCR mixture consisted of 0.2 µL of Phusion high fidelity (0.2 µL), 4 µL of high fidelity (HF) buffer (F520l; Thermo Scientific, Waltham, MA, USA), 0.4 µL of dinucleotide triphosphates (dNTPs), 1 µL of primers, 1.5 µL of template DNA (5 ng/µL), and 20 µL of H_2O. For the nodule fraction, 1 µM peptide nucleic acid (PNA) was used to target plastid (pPNA, 5′-GGCTCAA CCCTGGACAG-3′) and mitochondrial (mPNA, 5′-GG CAAGTGTTCTTCGGA-3′) DNA (PNA Bio, Newbury Park, CA, USA) as PCR clamps [44]. The PCR conditions were as follows: 98 °C for 1 min; 35 cycles of 98 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s; and a final elongation step of 72 °C for 7 min. Each DNA sample was amplified in triplicate, pooled, and then purified using a PCR clean-up kit (D4014, ZymoResearch). For each amplification run in 96-well plates, PCR-grade water was used as a negative control (no-DNA control). We proceeded with 16S rRNA gene diversity only because less than 1% of the samples were positive for ITS gene amplification for fungal community detection (data not shown). The samples were pooled and sequenced on the Illumina MiSeq platform via the V3 chemistry of 300PE run at M/s. Molecular Research DNA Laboratory in Texas, USA.

Processing of sequencing data

Initial quality filtering and read alignment were performed via USEARCH 10 fastq_mergepairs with fastq_maxee with an EE score of 1 [45]. After barcode removal, only reads above the desired length of 265 bp were used for further analysis. Reads were filtered from plant chloroplasts and mitochondria (approximately 2% of the initial reads were of plant origin) using a custom-made Bash script [46] (Additional File 3). The reads were binned into zero-radius operational taxonomic units (zOTUs), and chimeras were removed according to the Usearch10 pipeline with Unoise3 [47]. Bacterial zOTUs were annotated using the SILVA SSU132 16S rRNA database [48]. Additional details on the sequencing data processing, including quality control measures, read counts, and zOTU clustering procedures for all phases of this study, are provided in Additional File 2.

Bioinformatic and statistical analyses of microbiome

Comprehensive statistical, visual, and meta-analyses of the microbiome, including diversity analyses and comparisons along with graphical representation, were performed using MicrobiomeAnalyst [49, 50], an R-based online tool (https://www.microbiomeanalyst.ca/). The data were filtered for low count and low variance and normalized by cumulative sum scaling for marker gene (16S rRNA) analyses. The Shannon diversity index was used to measure the alpha diversity with the Mann–Whitney/Kruskal–Wallis (nonparametric tests) statistical method for significance testing. The Bray–Curtis index was calculated and visualized using principal coordinate analysis (PCoA) plots to assess beta diversity among the samples. The significance of the index was evaluated with permutational multivariate analysis of variance (PERMANOVA). The core taxa (phyla and genera) were visualized as heatmaps of compositional (relative) abundance. The key constituents of the core microbiome were identified at a relative abundance threshold of 0.01% and a sample prevalence of 20% [49, 50]. The unique and/or predictive features (biomarkers) were identified and classified using linear discriminant analysis (LDA) effect size (LEfSe) and random forest analysis. The classification errors (Out-of-bag [OOB] errors) were estimated to validate each random forest model.

The statistical significance of factors influencing the nodule microbial community was evaluated through permutations of residuals under a reduced model, the sum of squares type III (partial) with 9,999 permutations using unrestricted permutations of the raw data model of PERMANOVA. The *pseudo-F* values obtained from PERMANOVA served as proxies, indicating the relative importance of each factor in differentiating the samples. The *pseudo-F* values for each set of factors were plotted and visualized in Prism 9 (GraphPad, San Diego, USA).

Results

Influence of soil type and genotype on the pigeonpea nodule microbiome

The impact of soil type and genotype on the bacterial community associated with pigeonpea nodules was studied in three different pigeonpea genotypes grown in three distinct Indian soils. Nodules of different shapes, sizes, and quantities were obtained from pigeonpea plants (Supplementary Figs. S2-S3, Additional File 2).

The bulk soil fraction had a greater α-diversity (Shannon diversity index) than the pigeonpea nodules (Supplementary Fig. S4, Additional File 2). LEfSe analysis identified *Bradyrhizobium* in nodules as a biomarker associated with cultivated pigeonpea. Proteobacteria, Chloroflexi, and Planctomycetes were the predominant phyla in the nodules of pigeonpea. *Bradyrhizobium*, Unclassified_Roseiflexaceae,

Unclassified_Planctomycetales, Unclassified_Rokubacteriales, and Unclassified_Comamonadaceae were the most abundant taxa at the genus level in the pigeonpea nodule microbiome. Among the genotypes, the hybrid genotype MKK exhibited the highest α-diversity (Shannon diversity index), followed by Durga and Asha (Supplementary Figs. S5-S7, Additional File 2).

LEfSe analysis identified *Bradyrhizobium* as a biomarker for the Asha genotype. Differential abundance at the genus level was observed across the selected genotypes. *Bradyrhizobium*was the most abundant genus among all the genotypes, but it was highly abundant in the Asha genotype. The soil type also influenced bacterial diversity. Vertisols presented the highest α-diversity (Shannon diversity index), followed by Inceptisols and Alfisols, although the α-diversity in Vertisols was lower than that in pre-sowing soils. In Alfisols and Vertisols, the dominant phyla were Proteobacteria, Chloroflexi, and Methylomirabilota, whereas in Inceptisols, Proteobacteria, Planctomycetes and Chloroflexi were the most abundant. All soils were dominated by *Bradyrhizobium* at the genus level, with a greater abundance detected in Alfisols (Supplementary Figs. S8-S9, Additional File 2).

The core nodule microbiome of multiple pigeonpea genotypes grown in three different soil types was dominated by Proteobacteria, Chloroflexi, and Planctomycetes at the phylum level. The key constituents of the core nodule microbiome across all the soils at detection thresholds (relative abundances) ranging from 0.6 to 1% included *Bradyrhizobium*, Unclassified_Roseiflexaceae, Unclassified_Rokubacteriales, *Blastococcus*, Unclassified_Planctomycetales, Unclassified_Comamonadaceae, Unclassified_Acidobacteria, Unclassified_Pirellulaceae, and Unclassified_Nitrosomonadaceae (Supplementary Fig. S10, Additional File 2). Analysis of similarity (ANOSIM) values for all ordered groups were calculated on the basis of the Bray–Curtis similarity matrix and are depicted using PRIMER 7 (Supplementary Table S1, Additional File 2).

Edaphic factors shaping the pigeonpea nodule microbiome

Nodules from pigeonpea plants grown in laterite soil exhibited the highest α-diversity (Shannon diversity index), followed by those grown in black, red, alluvial, and mixed red and black soils (Supplementary Fig. S11, Additional File 2). LEfSe analysis revealed *Limnobacter* and *Flavisolibacter* as the differentially abundant bacterial genera in mixed black and red soils, and *Novosphingobium* in laterite soils. The bacterial genera *Ideonella*, *Azospirillum*, *Flavobacterium*, *Ensifer*, *Priestia*, and *Curvibacter* were identified as biomarkers for black soil; *Novosphingobium*, *Caenimonas*, and *Aureimonas* were identified for laterite soil; *Limnobacter*, *Flavisolibacter*, and *Herbaspirillum* were identified for mixed red and black soil; and *Chitinophaga* and *Pseudomonas* were identified for red soil. Amplicon sequencing revealed significant shifts in the relative abundance of these differentially abundant and biomarker bacterial genera identified by LEfSe and RF analyses (Supplementary Fig. S12, Additional File 2).

All the nodules across the soil types presented a greater relative abundance of several non-rhizobial endophytes (NREs), whereas the rhizobial taxa *Ensifer*, *Bradyrhizobium*, *Microvirga*, *Mesorhizobium*, and *Shinella* were less abundant (Supplementary Figs. S13-S14, Additional File 2). Nodules from plants grown in acidic soils presented the highest α-diversity (Shannon diversity index), followed by those from alkaline and neutral soils. LEfSe analysis identified *Novosphingobium*, *Caenimonas*, and *Pseudomonas* as differentially abundant in acidic soils and *Limnobacter* and *Flavisolibacter* as differentially abundant in alkaline soils. All the nodules, irrespective of the soil pH, presented greater relative abundances of several NRE taxa, whereas the rhizobial taxa *Ensifer*, *Bradyrhizobium*, *Microvirga*, *Mesorhizobium*, and *Shinella* were less abundant in the nodules. Notably, *Ensifer* had a greater relative abundance in alkaline soils than in acidic or neutral soils (Supplementary Figs. S15-S16, Additional File 2).

Nodules from plants grown in soils with moderate N levels (280–560 kg ha^{-1}) presented significantly greater α-diversity (Shannon diversity index) than those from soils with low N levels (< 280 kg ha^{-1}) did (Supplementary Figs. S17-S18, Additional File 2). Nodules from plants grown in soils with sufficient B concentrations (≥ 0.5 mg kg^{-1} [0.5 ppm]) presented significantly greater α-diversity than those from soils deficient in B concentrations (< 0.5 mg kg^{-1} [0.5 ppm]) (Supplementary Figs. S19-S20, Additional File 2). Among the nodules from plants grown in soils sampled from different geographical locations at varying altitudes (ranging from 50 m to 600 m above mean sea level), those from higher altitudes (≥ 300 m) exhibited slightly higher α-diversity than those from lower altitudes (< 300 m) (Supplementary Figs. S21-S22, Additional File 2). The α-diversity did not significantly differ between nodules from plants grown in soils with and without fertilizer application (Supplementary Figs. S23-S24, Additional File 2).

Among the nodules from plants grown in soils with different intercropping patterns, the pigeonpea–sunflower (*Helianthus annuus*) intercropping system exhibited the highest α-diversity, followed by the black gram (*Vigna mungo*), soybean (*Glycine max*), green gram (*Vigna radiata*), cotton (*Gossypium herbaceum*), horse gram (*Macrotyloma uniflorum*), and maize (*Zea mays*) intercropping systems. Solitary pigeonpea cropping resulted in greater α-diversity than intercropping with groundnut

(*Arachis hypogaea*), guava (*Psidium guajava*), sorghum (*Sorghum bicolor*), cucumber (*Cucumis sativus*), or ridge gourd (*Luffa acutangula*). *Ensifer* was more abundant in the bulk soil, whereas *Pseudomonas* was relatively abundant in nodules across multiple intercropping systems (Supplementary Figs. S25-S26, Additional File 2).

Analysis of the core microbiome enabled the identification of core taxa that remained unchanged in composition across different sample groups on the basis of sample prevalence and relative abundance. Pigeonpea nodules were colonized primarily by Proteobacteria and Bacteroidetes (Fig. 1A). The main bacterial genera found in pigeonpea nodules included *Limnobacter*, *Novosphingobium*, *Flavisolibacter*, *Caenimonas*, *Pseudomonas*, *Chitinophaga*, *Ensifer*, Unclassified_Aurantimonadaceae, *Bradyrhizobium*, and *Microvirga*. These genera constituted the core nodule microbiome across all the studied soils, with detection thresholds (relative abundance %) ranging from 0.010 to 0.728 (Fig. 1B).

Multifactor PERMANOVA analysis revealed that soil type, pH, macronutrient, and micronutrient levels were the most significant factors influencing the bacterial community composition in pigeonpea nodules ($p < 0.05$) (Fig. 2A–E and Supplementary Table S2, Additional File 2). Geoclimatic factors and agricultural practices also had notable but lesser impacts. The results regarding the influence of different factors, which were not included above, are provided in Supplementary Figs. S27-S56 (Additional File 2).

Single-nodule microbiomes of pigeonpea genotypes: spatial distribution across primary, secondary, and tertiary roots

The bulk soil exhibited significantly greater α-diversity (Shannon diversity index) than the nodules, regardless of

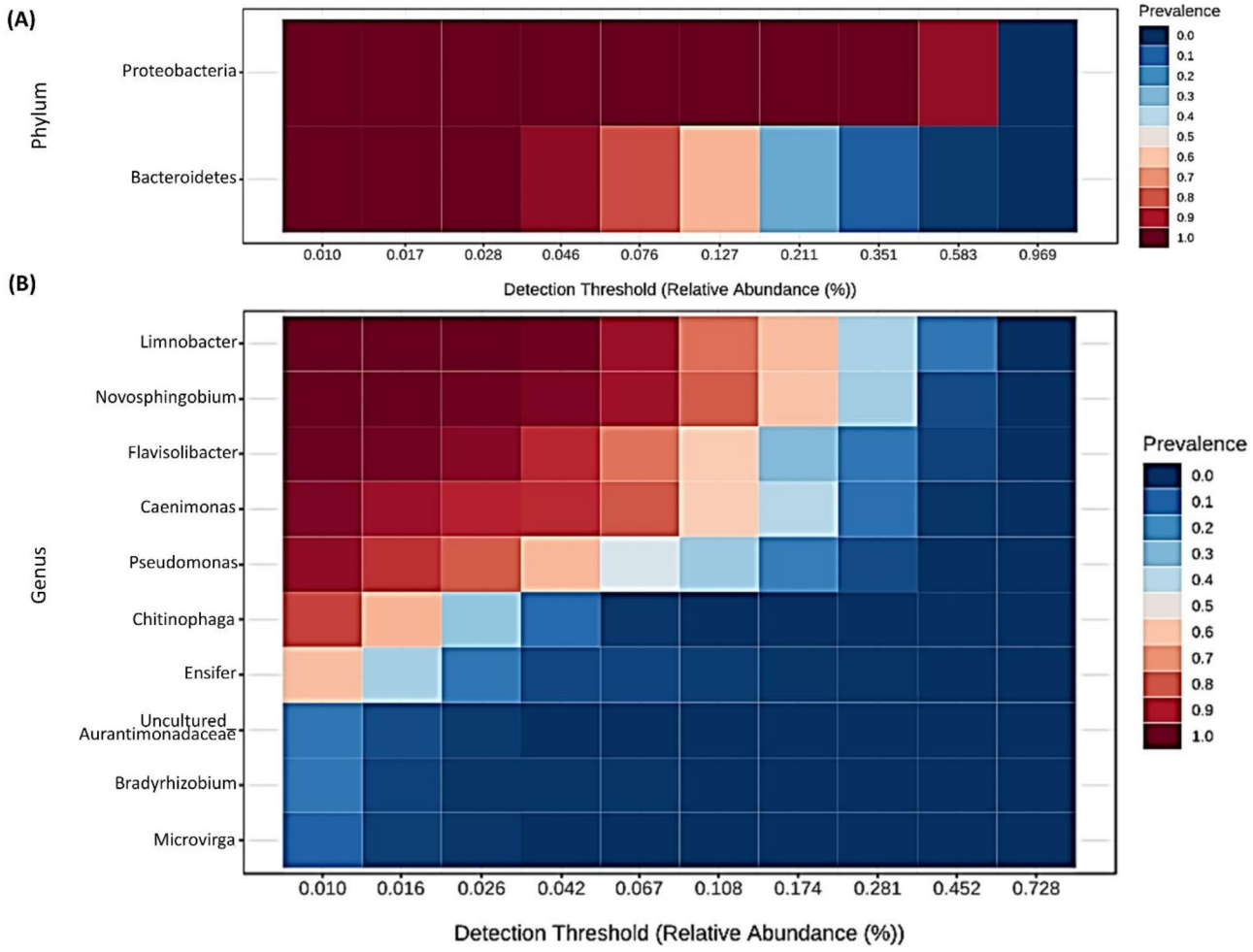

Fig. 1 Heatmaps representing the core nodule microbiome of pigeonpea across diverse soils at the (**A**) phylum and (**B**) genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation in the prevalence of each phylum/genus is indicated by a color gradient from blue (decreased) to red (increased). The 'Uncultured_' taxa label in the figure represents unclassified bacterial taxa

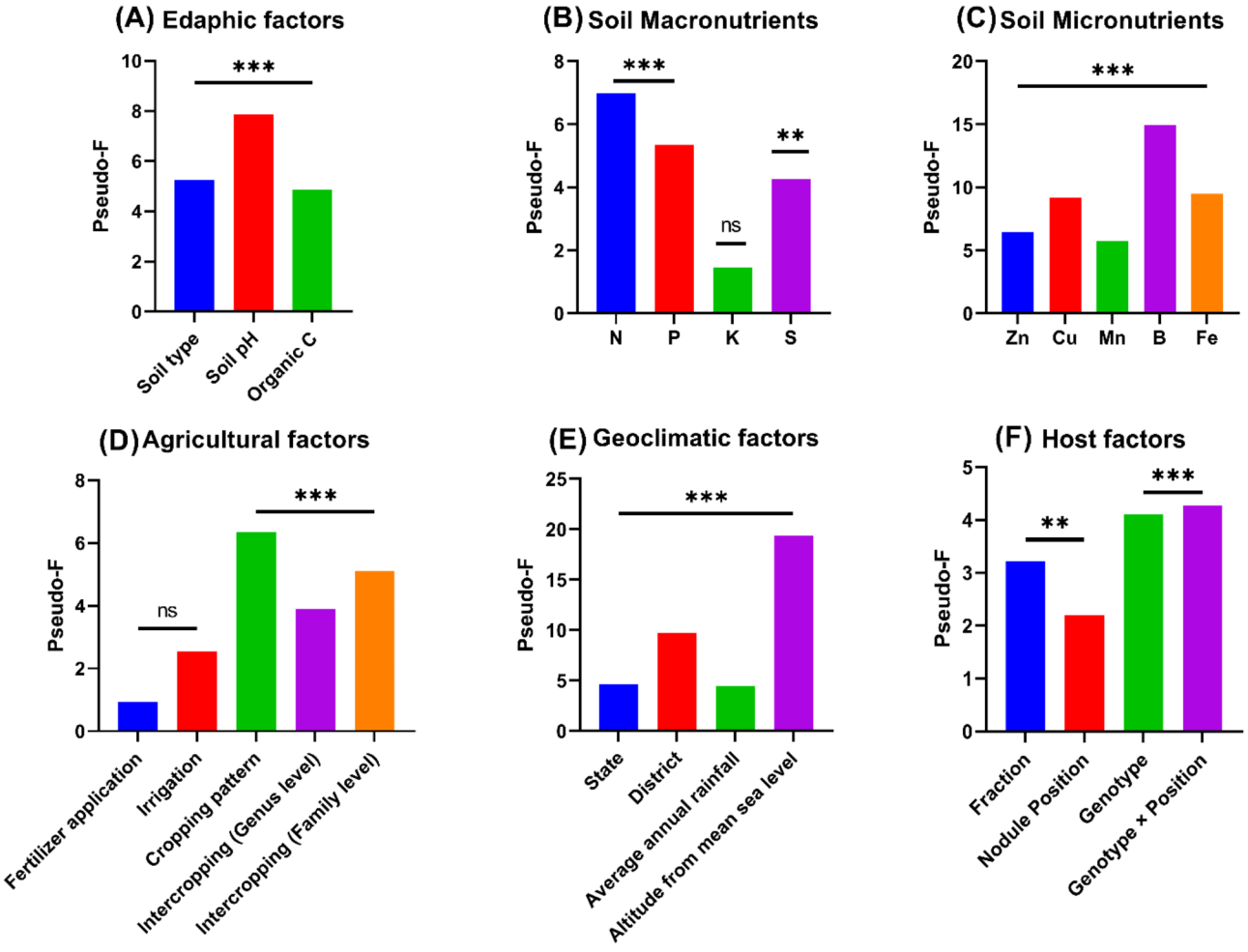

Fig. 2 PERMANOVA output measuring the influence of different factors on the pigeonpea nodule microbiota using the *pseudo-F* values as proxies. (**A**) Edaphic factors, (**B**) Soil macronutrients, (**C**) Soil micronutrients, (**D**) Agricultural factors, (**E**) Geoclimatic factors, and (**F**) Host factors. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$

their position on the roots. Among the nodule positions, primary and tertiary root nodules presented very similar α-diversity, with primary root nodules having a marginally higher Shannon index than did tertiary root nodules. In contrast, secondary root nodules had reduced α-diversity. The PCoA plots revealed distinct clustering patterns for each group, and PERMANOVA indicated significant differences in zOTU assemblage between nodule bacterial communities on the basis of their position on the roots. All the nodules presented a relatively high abundance of the rhizobial genera *Bradyrhizobium* and *Rhizobium*. Other rhizobial taxa, such as *Shinella*, *Mesorhizobium*, *Microvirga*, and *Ensifer*, were also abundant in the nodules, although to a lesser extent. The taxonomic composition of the nodule bacterial communities was influenced by both the host genotype and the position of the nodule on the roots (Supplementary Figs. S57-S60, Additional File 2).

Multifactor PERMANOVA analysis (Supplementary Table S3, Additional File 2) revealed that host genotype (*pseudo-F* = 4.1087) exerted the strongest influence on the nodule bacterial community composition, indicating greater variation between sample groups than within each group. This was followed by the influence of plant fraction (nodule and bulk soil; *pseudo-F* = 3.2245) and nodule position on the root (*pseudo-F* = 2.1938) (Fig. 2F). The interaction between plant genotype and nodule position had a significantly stronger influence than the individual factors did (*pseudo-F* = 4.2787).

ANOSIM revealed significant differences in the overall nodule bacterial community structures across pigeonpea genotypes and nodule positions. Among the genotypes, the most pronounced community variations were observed between MKK and Durga (ANOSIM $R = 0.499$, $P < 0.001$), followed by Durga and Asha ($R = 0.306$, $P < 0.05$), whereas there was less difference between Asha and MKK ($R = 0.248$, $P < 0.01$). Significant

bacterial community variation was also detected within each genotype across different nodule positions (primary, secondary, and tertiary roots) (Supplementary Table S4, Additional File 2). A schematic representation of the variation in nodule microbial community composition between nodule positions across the genotypes (based on ANOSIM *R* values) is presented in Fig. 3.

Nodule microbiome of wild pigeonpea (*Cajanus scarabaeoides*)

The core nodule microbiome of the wild pigeonpea (Supplementary Fig. S61, Additional File 2) was predominantly composed of Proteobacteria, Methylomirabilota, and Actinobacteria at the phylum level, with *Bradyrhizobium* being the most prevalent at the genus level (Fig. 4). A diverse range of rhizobia and NREs were observed in the core microbiome.

Comparative analysis of the nodule microbiomes of wild and cultivated pigeonpea genotypes using ANOSIM (Table 1) revealed significant differences in the bacterial community structure, with the wild genotype exhibiting a distinct microbiome compared to its cultivated counterpart. The most significant difference of 85.1% was observed between the wild and cultivated genotypes, with a total difference of 66.1% at $p < 0.001$. Additionally, a considerable difference of 35.3% was observed between the control and the wild type, which was still significant at $p < 0.05$. Principal coordinate analysis further supported the ANOSIM results, indicating that 28.2% of the variation in the bacterial communities was explained by the X-axis and 11.9% by the Y-axis (Fig. 5).

Discussion

Nitrogen-fixing bacteria form symbiotic partnerships with leguminous plants (and non-legume *Parasponia* spp.) in nodules, where atmospheric nitrogen is reduced to ammonia, promoting plant growth and development. Plants exchange photosynthetic assimilates in the form of organic carbon to provide nutrients and support symbiotic bacteria, apart from the niche. They secrete

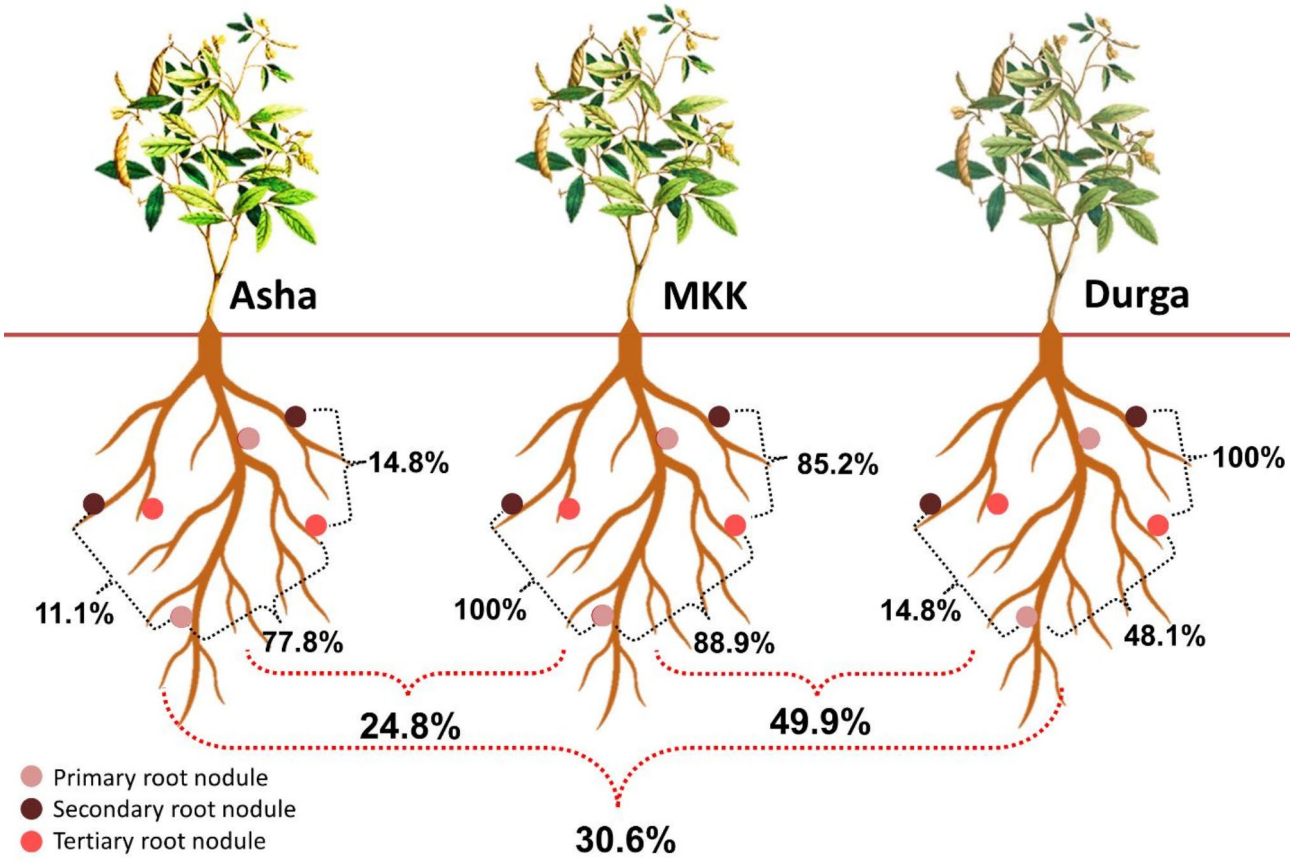

Fig. 3 Schematic representation of analysis of similarities (ANOSIM) between the pigeonpea nodule microbial communities across the three different genotypes. Numerals represent the percentage of variance in the nodule microbial community composition explained by the analyzed factors based on ANOSIM *R* values. The dotted lines in black represent the differences between nodule positions within each genotype, while the red dotted lines represent the overall variation in nodule microbial communities between the genotypes. Higher percentages indicate a stronger influence of the respective factor (nodule position or genotype) on the microbial community composition. The distances depicted by the dotted lines are representative and do not correspond to actual spatial distances in relation to the percentage variations

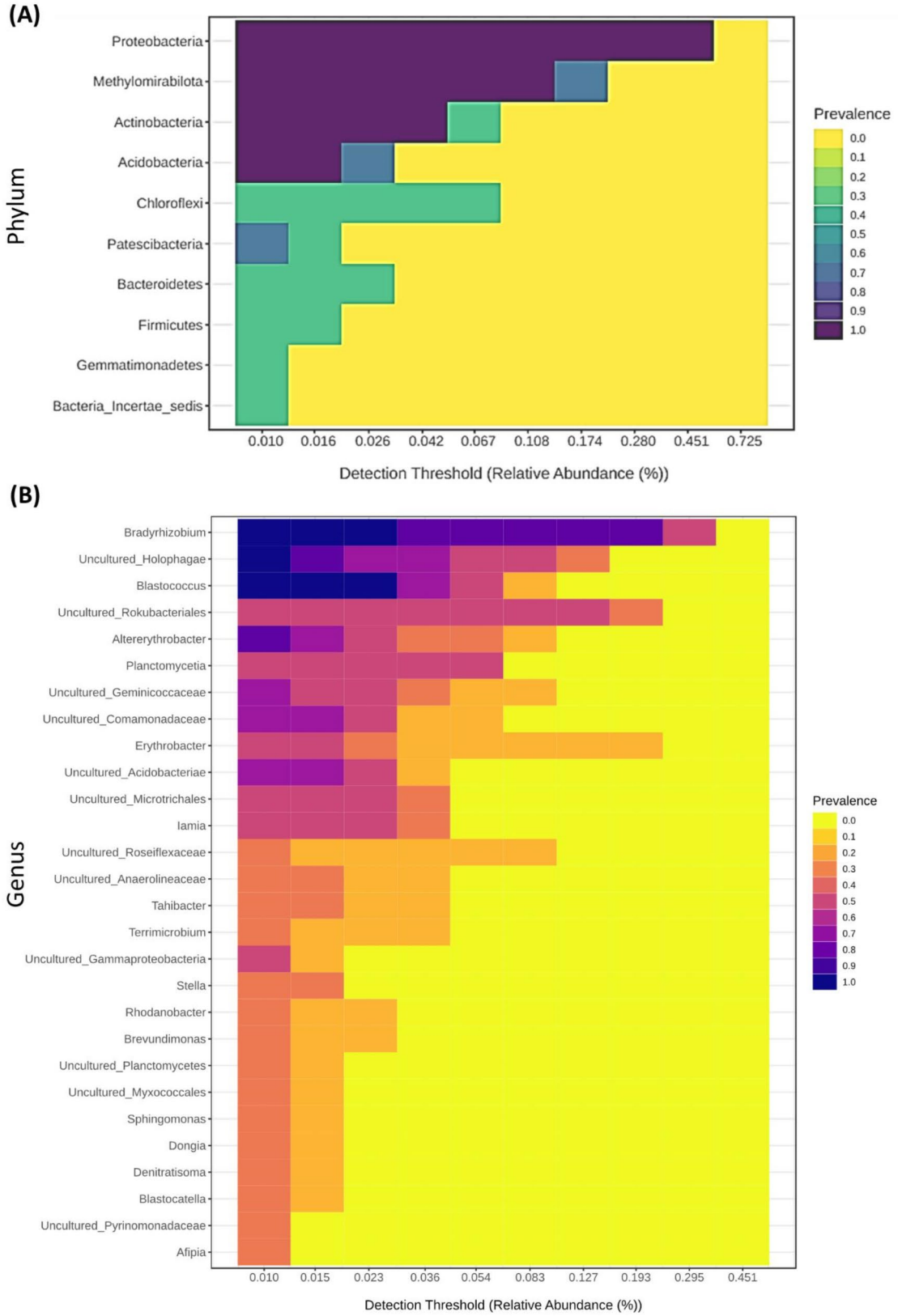

Fig. 4 The core microbiome of wild pigeonpea nodules at the (**A**) phylum and (**B**) genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation in the prevalence of each phylum/genus is indicated by a color gradient from blue (increased) to yellow (decreased). The 'Not_assigned/Uncultured_' taxa label in the figure represents unclassified bacterial taxa

Table 1 ANOSIM differences of the nodule microbiome between pigeonpea genotypes. Comparative differences were calculated using a Bray–Curtis similarity distance matrix by ANOSIM (PRIMER 7 software).

Groups	*R*	*P* value
Cultivated, control	59.2	0.001
Cultivated, wild	85.1	0.001
Control, wild	35.3	0.042
Overall	66.3	0.001

flavonoids into the rhizosphere to recruit these rhizobia, leading to signal exchange, nodulation, and symbiosis [51, 52]. This symbiotic interaction occurs only between specific legume(s) and their corresponding symbionts(s).

Globally, pigeonpea and other important legume crops are rich sources of protein, nutrients, and other benefits to the rapidly increasing human population while being more sustainable than most other crops are [3]. Rhizobial endosymbionts associated with pigeonpea nodules supply the nitrogen required for plant growth [14]. Notably, legume root nodules host a diverse community of bacteria rather than a single species of rhizobia [10]. Pigeonpea nodules harbor diverse rhizobia and non-rhizobial endophyte (NRE) taxa [21, 25, 26].

The nodule bacterial community is shaped by the host genotype and nodule position on the root

Our results demonstrated considerable differences in the overall nodule bacterial community structure across the three genotypes and between nodule positions within each genotype. The nodule microbiota was similar at the phylum level but more distinct at the genus level in terms of the relative abundance of each taxon. The variation in the nodule bacterial community structure across various nodule positions within individual genotypes could be due to a random process since close root nodules show significant differences in their microbiome composition [53]. The host genotype can also influence the nodulation process [54].

Culture-dependent studies have shed light on the potential benefits of NREs on their host plants [53, 55–57]. However, their preferential selection by legume hosts is not known. Culture-independent (metagenomic) studies have demonstrated the vast diversity and abundance of NREs in legume nodules [53, 55, 58]. Our study found that many non-rhizobial taxa were relatively abundant in the nodules. It is unclear whether their presence is linked to potential biological and/or biogeochemical cues resulting from the tripartite interactions between the soil, host plants, and indigenous microbiota.

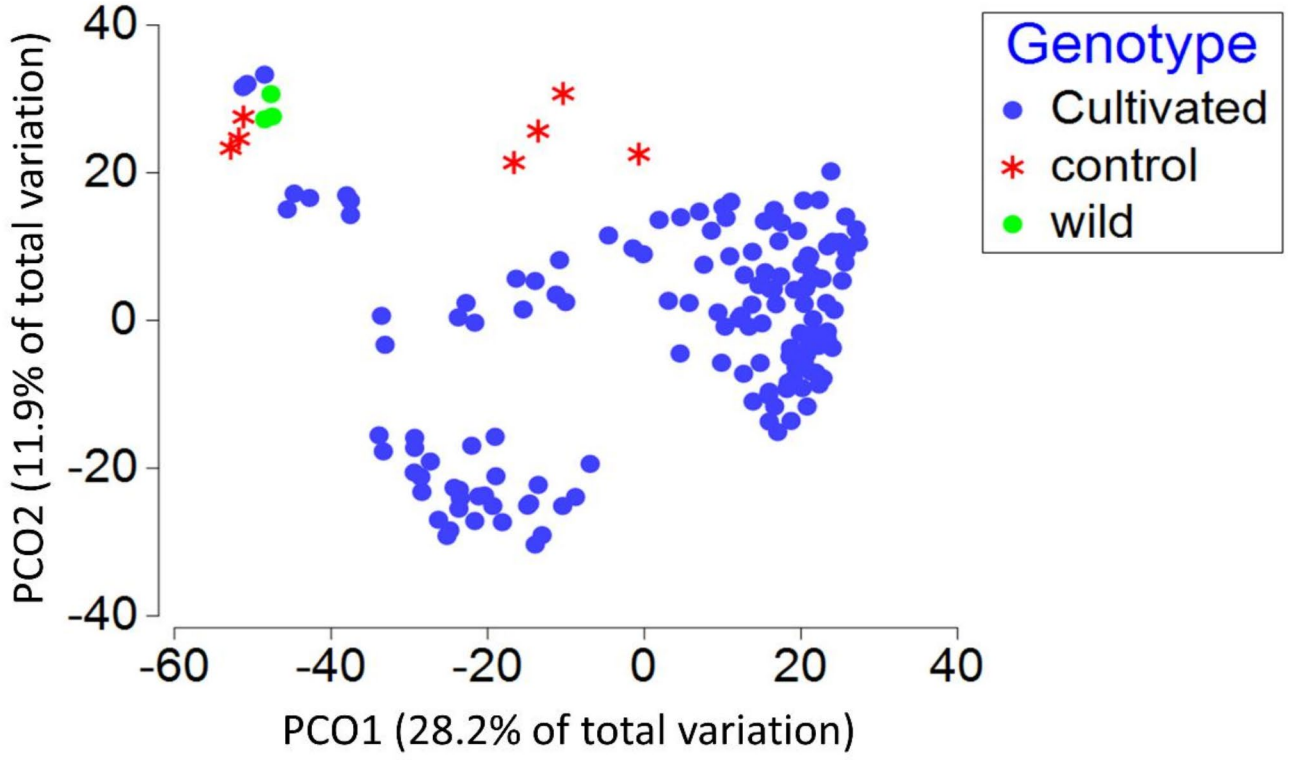

Fig. 5 Principal coordinate analysis for the wild vs. cultivated pigeonpea nodule microbiome. The samples were transformed by the square root and distance matrix constructed with the Bray–Curtis similarity metric. Total variations of 28.2% (X-axis) and 11.9% (Y-axis) were observed with principal coordinate analysis

Edaphic and agro-climatic factors shape the Pigeonpea nodule bacterial community

Soil parameters and history are key drivers of microbial composition and diversity. Soil type, and to a lesser extent, host genotype, influence the abundance and structural and functional diversity of soil and root-associated microbial communities [31, 59–61]. We found that edaphic factors play a significant role and explain most of the variation in the bacterial community structure of the pigeonpea nodule microbiome. Variations in soil type, pH, and nutrient status were strongly associated with differences in nodule bacterial diversity and abundance, confirming previous studies that highlighted the influence of soil properties and host genotype on the composition of root-associated microbiomes [10, 62–68].

Our findings also indicate that agricultural practices significantly affect the nodule microbiome. Agricultural practices significantly influence the diversity, abundance, and richness of the bacterial community in the pigeonpea rhizosphere and drive the dynamics of dominant rhizobacterial taxa [69]. Although fertilizer amendments are known to influence the soil properties and microbial community composition [70–74], we did not observe a significant effect of fertilizer amendment (in the soil) on the pigeonpea nodule microbial community. However, significant variation in the relative abundance of the nodule microbiota was observed across the different cropping systems, particularly intercropping, likely due to increased root proximity and altered root exudate profiles that promote beneficial microbial recruitment [72, 75–79].

Additionally, climatic factors such as rainfall [62, 63] and geographical factors, including altitude and location [80–82], were correlated with variations in the nodule microbiome. Notably, location-driven biogeographical patterns in nodule bacterial communities, previously documented in soybean [83, 84], were also evident in pigeonpea, underscoring the complex interplay of environmental and agronomic factors in modulating these communities.

Pigeonpea nodules are colonized by Proteobacteria and Bacteroidetes

Bulk soil exhibited greater microbial diversity than pigeonpea nodules, as reflected by the α-diversity and bacterial community composition data. The soil serves as a reservoir or meta-community for microorganisms that comprise the nodule microbiomes. Our findings align with those of Miranda-Sánchez et al. [85] and Xiao et al. [55], who reported that legume hosts recruit nodule endophytes from bulk soil through a hierarchical filtering process, wherein a gating mechanism at the root possibly restricts the microbiota from entering the nodule endosphere. Root-associated microbiomes, especially nodule microbiomes, are typically less diverse than soil microbiomes because of plant selection and competition among microorganisms for plant-derived resources [51, 55]. Nevertheless, nodule microbiomes can still be extremely complex, containing hundreds of distinct bacterial species [86].

Pigeonpea nodules showed a specific selection for Proteobacteria and Bacteroidetes, regardless of geographical origin, soil nutrient status, soil type, or soil history. Members of these phyla are often abundant in plant tissues [87, 88]. Proteobacteria, especially Alphaproteobacteria, are common root colonizers across multiple soils and plant species, including barley, rice, *Arabidopsis*, and *Lotus* [89–92]. Alphaproteobacteria members likely metabolize plant-derived nutrients quickly [88] and share genomic similarities with plant symbionts [93].

The main nodule-inhabiting genera representing the core microbiome across diverse soils were *Limnobacter*, *Novosphingobium*, *Flavisolibacter*, *Caenimonas*, *Pseudomonas*, *Chitinophaga*, *Ensifer*, *Bradyrhizobium*, and *Microvirga*. This suggests that these genera are particularly attracted to pigeonpea roots, possibly through active plant secretion. Plants influence the microbiota around their roots, creating a gradient of impact that decreases with increasing distance from the roots [46]. While plant presence shapes the surrounding microbial community, other factors, such as soil type, determine its exact profile.

Diverse rhizobia are associated with pigeonpea nodules

We consistently detected at least three rhizobial genera across all pigeonpea nodules: *Ensifer*, *Bradyrhizobium*, and *Microvirga*. In contrast, *Mesorhizobium* and *Shinella* were less abundant. Among these, *Ensifer* was the most abundant nodule-colonizing rhizobial genus, as identified through our 16S rRNA gene amplicon assay. *Ensifer* spp. are common endosymbionts of native Indian legumes that grow in alkaline soils [94–96]. We observed a relatively high abundance of *Ensifer* in the nodules of pigeonpea grown in alkaline soils, suggesting that *Ensifer* might outcompete *Bradyrhizobium*, the most common pigeonpea endosymbiont [14, 21], in these native soils to colonize pigeonpea nodules. Moreover, the case of pigeonpea is not limited, as soybean roots, which typically host *Bradyrhizobium*, are colonized by a bacterial community where this symbiont constitutes merely about 1% of the total population [60, 97]. This relatively low symbiont abundance contrasts with that of other legumes, such as pea, where the symbiotic genus *Rhizobium* accounts for approximately 10–20% of the root microbiome; *Medicago*, with *Ensifer* accounting for approximately 10–60%; and *Lotus*, where *Mesorhizobium* accounts for approximately 10% of the root-associated bacteria [91, 98–101].

Bradyrhizobium, abundant in Indian soils [14], poorly colonizes pigeonpea roots under competition from other soil bacteria and likely includes many non-symbiotic strains. Nevertheless, pigeonpea plants can still nodulate [46]. *Bradyrhizobium* has evolved to be recognized by pigeonpea, infect its roots, and develop root nodules. *Mesorhizobium* association with pigeonpea nodules has been well documented in Indian soils [15, 17]. Nevertheless, the detection of zOTUs belonging to atypical rhizobia, such as *Shinella* and *Microvirga* in pigeonpea nodules, which are potential endosymbiotic bacteria associated with other legumes, indicates that these atypical taxa can be endophytes in non-host legumes such as pigeonpea [55, 102].

The core nodule microbiome of wild pigeonpea is dominated by *Bradyrhizobium*

Cajanus scarabaeoides (L.) Millspaugh is one of the few wild relatives of pigeonpea that can reproduce. It is highly tolerant to drought and salinity, is mostly resistant to insect pests, and has a high protein content in its grains [103]. Widely distributed in South Asia, this species has been largely overlooked. Unlike contemporary agricultural systems, which are artificially molded by human intervention and rely on fertigation and chemigation to maintain high yields, wild ecosystems offer greater genetic variety, soil heterogeneity, interspecies competition, and biodiversity [104]. Modern technology allows the creation of high-yielding varieties that incorporate microbiota from their wild relatives [105]. However, plant–soil feedback in agricultural environments, particularly the effects of plant domestication on soil ecosystems and geochemical processes, remains underexplored.

We found that *Bradyrhizobium* dominates the core microbiome of wild pigeonpea nodules. *Bradyrhizobium* is associated with pigeonpea and other legumes such as cowpea, black gram, and green gram [106, 107]. In the Dominican Republic, *Bradyrhizobium* strains form a nitrogen-fixing symbiosis with pigeonpea and are effective biofertilizers to replace N fertigation [19]. *Bradyrhizobium* sp. is associated with high pigeonpea yields in Côte d'Ivoire [21]. Biological nitrogen fixation in groundnut was improved by *B. yuanmingense* isolated from Ghanaian native soils [108]. Similarly, pigeonpea performance was enhanced by inoculation with biochar-formulated *Bradyrhizobium* strains [109]. In our previous work, we observed poor competitiveness of *Bradyrhizobium* toward the cultivated pigeonpea root microbiome, as both cultivated and wild pigeonpea nodules were dominated by *Bradyrhizobium*, revealing specific recruitment by all genotypes [46]. This selective recruitment ensures that the plant prioritizes microbial partners that provide essential functions, such as nitrogen fixation, even if these partners are not the most competitive in the broader soil environment.

Domestication significantly alters plant physiology and microbiomes [29, 110, 111]. The microbiota associated with the nodules of wild legumes are phylogenetically diverse and often possess various plant growth promoting abilities, including osmotolerance [112]. They may also represent unique natural hotspots of antibiotic-resistance genes [113]. Interestingly, a study on nodule bacterial diversity conducted in continental Portugal with 16 wild legume species revealed that most isolated strains belong to non-rhizobial genera like *Pseudomonas* and *Flavobacterium*, rather than typical rhizobia [112]. Wild soybeans, which are ecologically more resilient than cultivated soybeans, may have evolved to recruit beneficial bacteria in their rhizosphere that may increase nutrient uptake, biostasis, and disease resistance [114]. These findings have important implications for understanding legume–rhizobia interactions and optimizing symbiotic nitrogen fixation in agriculture.

Limitations of the study

Our study primarily focused on spatial variation in the pigeonpea nodule microbiome. We did not extensively analyze temporal dynamics and seasonal variations in microbial community composition, which could influence symbiotic interactions, microbial succession, and stability. Additionally, the study's focus on specific genotypes and soil types limits the broader applicability of the findings across diverse agroecosystems. The research was limited to four genotypes, including three cultivated varieties and one wild relative (*Cajanus scarabaeoides*), while many pigeonpea cultivars and wild accessions remain unexplored. Future studies should include these wild accessions, modern hybrids, and landraces, which may harbor unique microbial associations beneficial for crop resilience and symbiotic nitrogen fixation. Although edaphic factors were considered, other influences, such as plant physiological status and root exudate composition, were not examined. Addressing these gaps in future research could provide a more comprehensive understanding of the factors shaping the pigeonpea nodule microbiome.

Conclusions

In conclusion, this study elucidates the complex interactions between host genotype, nodule position, and edaphic factors in shaping the nodule microbiome of pigeonpea. Our findings reveal that soil properties, particularly pH, nutrient status, and soil type, exert a substantial influence on the nodule bacterial community than the host genotype, underscoring the critical role of environmental conditions in symbiotic relationships. The core nodule microbiome, dominated by Proteobacteria

and Bacteroidetes, with *Bradyrhizobium* and *Ensifer* as the major rhizobial taxa and various non-rhizobial taxa, reflects the selective recruitment by the host plant. While bulk soil exhibited a higher microbial diversity, the nodule microbiome was less diverse but highly specialized, indicating a selective process driven by plant-microbe interactions. Comparative analysis between wild and cultivated pigeonpea revealed substantial differences in their nodule microbiomes, underscoring the influence of domestication on microbial community composition. These insights into the microbial diversity and community structure of pigeonpea nodules provide a foundation for developing strategies to boost crop productivity and resilience through targeted microbial management. Future research, with a focus on the functional characterization of these microbial communities and their interactions with the host plant, would help to exploit microbial potential in sustainable agriculture.

Abbreviations

ANOSIM	Analysis of similarity
LDA	Linear discriminant analysis
LefSe	Linear discriminant analysis (LDA) effect size
MKK	Mannem Konda Kandi (ICPH–2740)
NRE	Non–rhizobial root nodule endophytes
OOB	Out–of–bag
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
PNA	Peptide nucleic acid
zOTU	Zero–radius operational taxonomic unit

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-025-00707-4.

Additional File 1: Soil characterization data.

Additional File 2: Detailed processing of sequencing data across the various experimental phases; Supplementary Figures S1–S61; Supplementary Tables S1–S4.

Additional File 3: Bash code, merging report, and final reads per sample for all samples.

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Author contributions

ARP conceived and planned the study; AB, DC, and SPVSRN designed the study, conducted the experiments, and analyzed the data; SU, VRC, and PLC conducted the experiments; AB, DC, and SPVSRN drafted the manuscript; ARP reviewed and revised the manuscript. All authors have read, critically revised, and approved the final version of the manuscript.

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Data availability

The datasets generated and/or analysed during the current study are available in the SRA database of NCBI under BioProject accession number PRJNA1171966.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Clinical Trial Number

Not applicable.

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