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The invasive mimosoid legume *Dichrostachys cinerea* (L.) Wight & Arn is nodulated by diverse strains of *Ensifer* and *Bradyrhizobium* in different agroclimatic regions of India

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Abstract

Dichrostachys cinerea (L.) Wight & Arn, which belongs to the Mimosoid clade of the legume subfamily Caesalpinioideae, was introduced into India and has since become invasive across wide areas of the country. It is nodulated, and like all other mimosoids it has indeterminate nodules with its microsymbionts housed in membrane-bound symbiosomes rather than within cell wall-enclosed fixation threads. Fifty-eight bacterial strains were isolated from root nodules on plants growing in soils from 13 sampling sites in India with various agroclimatic conditions. Genetic analysis of 36 strains resulted in diverse RAPD genotypes, with equal composition of *Ensifer* and *Bradyrhizobium* as its root nodule microsymbionts. Multi locus sequence analysis (MLSA) of 12 strains using the *recA*, *glnII*, *atpD* and 16S rRNA genes revealed significant genetic diversity forming novel clades and lineages and are potential new species. The *D. cinerea* strains were variants of local symbionts previously described as rhizobia associated with native and exotic mimosoid trees, as well as rhizobia associated with the non-mimosoid Caesalpinioid shrub *Chamaecrista pumila* and wild Papilionoid legumes from India. The symbiosis essential genes (*nodA* and *nifH*) of the *D. cinerea* strains were diverse and clustered according to geographical origin. Mosaic combinations of core and *sym* genes were harbored by both *Ensifer* and *Bradyrhizobium* suggesting gradual diversification and microevolution of rhizobia under pressure from the host in combination with edaphic and environmental factors. The dominant microsymbionts of native and invasive legumes, including *D. cinerea*, in alkaline soils of India are essentially of the '*E. aridi*' and *B. yuanmingense* types. *Dichrostachys cinerea* rhizobia were symbiotically efficient on their homologous host, but also have ability to nodulate the crop *Vigna radiata*, and hence may be good candidates to be used for inoculants on legume crops as well as on Mimosoid trees (*P. cineraria*, *V. nilotica*, *V. raddiana*, *S. senegal*) used in sustainable agroforestry practices to enhance soil nitrogen content.

Keywords *Dichrostachys cinerea* · Root nodule · *Ensifer* · *Bradyrhizobium* · Concatenated phylogeny · Symbiotic genes

1 Introduction

Dichrostachys is a genus in the Mimosoid clade which is nested within the legume subfamily Caesalpinioideae (LPWG 2017; Sprent et al. 2017; de Faria et al. 2022). It comprises 14 species of shrub or small tree and is indigenous to the Old World tropics (Sprent 2005). The species *Dichrostachys cinerea* (L.) Wight & Arn., commonly known as the sickle bush, is a perennial tree native to Africa and Australia (https://www.cabidigitallibrary.org/doi/https://doi.org/10.1079/cabicompendium.18119). From Africa it was introduced in Cuba in the nineteenth century, later invading unused agricultural lands. It is also invasive in southern African savannas through clonal spread by root suckers (Nielsen et al. 2013; Wakeling and Bond 2007). It

One sentence summary: The invasive mimosoid legume *Dichrostachys cinerea* is promiscuous and nodulates in alkaline and acidic soils of India.

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is a drought-tolerant plant and can grow in soil with varying pH (4.5–8.5), it grows well on deep, sandy loamy soil, and can survive mean annual rainfalls from 200–400 mm (Coates-Palgrave 1988). Although *D. cinerea* is an aggressive and devastating invasive species in some countries it has utility e.g. it can be used as biomass for bioenergy generation. It also has several environment-related benefits, such as soil conservation, sand dune stabilization, re-vegetation, and intercropping, and hence it can provide a base for environmentally and economically sustainable agroforestry and silvo-pastoral systems (Heuzé et al. 2015; Sáez and Alfayate 2020). Like most Mimosoids, its capacity for root nodulation means it is able to symbiotically fix atmospheric nitrogen (N), allowing it to be used in co-cultivation systems as a source of N fertilization with other (non-legume) fodder or cash crops (Pule-Meulenberg and Dakora 2009; Nielsen et al. 2013).

In India *D. cinerea* is naturalized in dry deciduous forests in western to southern parts of the country including the states of Andhra Pradesh, Delhi, Haryana, Goa, Gujarat, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Orissa, Punjab, Rajasthan, Tamil Nadu and Uttar Pradesh (Bhandari 1990, CABI Compendium https://doi.org/10.1079/cabicompendium.18119). It was originally planted along with other legumes and grasses for the restoration of degraded land with reduced organic matter and poor moisture retention capacity e.g. Asola-Bhatti Wildlife Sanctuary situated in Delhi (Pant and Pant 2017). Little is known about the rhizobia that nodulate *D. cinerea*, but effective root nodules on *D. cinerea* were formed by *Rhizobium* sp. strain NGR234 (now named *E. fredii* NGR234) isolated from nodules of *Lablab purpureus* in Papua New Guinea (Trinick 1980), and by *R. fredii* USDA257 (now named *E. fredii* USDA257) isolated from wild *Glycine soja* in China (Pueppke and Broughton 1999). However, it failed to nodulate with the promiscuous Beta-rhizobium strain, *Paraburkholderia phymatum* STM815^{T} (Moulin et al. 2014). We have not encountered any published study related to the characterization of N-fixing rhizobia naturally associated with it in India or elsewhere. Therefore, the aim of present study was to isolate and characterize the root nodule bacteria (RNB) associated with *D. cinerea* in different agroclimatic zones of India specifically from soils of Rajasthan (RJ), Haryana (HR), Punjab (PB), Madhya Pradesh (MP), Puducherry (PY) and Meghalaya (ML). It was also considered to be of interest to compare its microsymbionts with those from native (*Mimosa hamata*, *M. himalayana*, *Prosopis cineraria*, *Vachellia jacquemontii*, *V. nilotica*, *V. leucophloea*, and *Senegalia senegal*) and invasive (*M. pudica*, *Leucaena leucocephala*) Mimosoid species in the Thar desert as well as from other parts of India, wherein most of the aforementioned genera were predominantly nodulated by diverse species of *Ensifer* (Gehlot et al. 2012, 2013, 2016; Sankhla et al. 2017; Choudhary et al. 2017, 2018, 2020; Chouhan et al. 2022). In consideration of its invasiveness, this investigation was initiated with the possibility that like other invasive legumes, such as *L. leucocephala* (Chouhan et al. 2022), the invasiveness of *D. cinerea* is assisted by an ability to nodulate with a broad diversity of native microsymbionts (species of *Ensifer*, *Bradyrhizobium*, *Rhizobium* and *Mesorhizobium*) similar to those previously reported from a wide range of native or exotic legumes growing in the Thar desert and other agroclimatic regions of India. Or, that it may have a specific preference in its selection of microsymbionts, and as with another invasive legume, *M. pudica* (Gehlot et al. 2013), its preferred symbiont has accompanied it from its native environment.

2 Materials and Methods

2.1 Trapping of rhizobia in root nodules of Dichrostachys cinerea from soils of various sites

Healthy seeds of *D. cinerea* (collected from plants growing in the Botanical Garden, J.N.V. University, Jodhpur) were surface sterilized by washing in 90% (v/v) alcohol and an antifungal agent [0.1% (w/v) Bavistin^{R}] for 1 min followed by five rinses in sterile distilled water. Seeds were further surface sterilized in 4% (v/v) sodium hypochlorite (NaOCl) for 1 min and washed 6 times with autoclaved distilled water to remove the residues of sterilizing chemicals. Surface sterilized seeds of *D. cinerea* were germinated on moist sterile filter paper and sowed in sterile pots filled with soils collected from different sampling sites in RJ, HR, PB, MP, ML and PY (Table 1, Fig. 1). Pots were placed in a greenhouse under controlled conditions (28–30 °C and natural sunlight giving 14 h light and 10 h of darkness) and moistened regularly with autoclaved tap water. After 8 to 10 weeks plants were harvested, and nodulation was checked as described earlier (Sankhla et al. 2017).

2.2 Nodule sampling for microscopy, and isolation and purification of rhizobia

From each harvested *D. cinerea* plant 2–3 root nodules were selected for rhizobial isolation, while other nodules were fixed in glutaraldehyde and prepared for light and transmission electron microscopy (TEM) according to de Faria et al. (2022). For isolation of rhizobia, adhered soil particles were removed with water and prior to surface sterilization they were wrapped in muslin cloth; the nodules were then surface-sterilized firstly in 90% (v/v) alcohol for 1 min, transferred to 0.1% (w/v) Bavistin^{R} for 30 s, and rinsed 4–5 times in autoclaved distilled water to remove traces of fungicide. Finally, the nodules were transferred to 4% (v/v) sodium

Table 1 Soil sampling sites with geographical coordinates, soil pH and list of strains isolated and purified from root nodules of *Dichrostachys cinerea*

	Soil sampling sites	District	State/Union territory of India and abbreviation used	Geographical coordinates (https://www.latlong.net)	Soil pH	No. of bacterial strains isolated from each site (Total 58)	Strain designation and molecular analysis (Out of total 58 bacterial strains 36 were genetically analysed based on DNA fingerprinting)
1.	Pokhran	Jaisalmer	Rajasthan (RJ)	26°55'28.48"N/71°54'58.49"E	8.4	4	**DC-RJ2**, DC-RJ3, DC-RJ4
2.	Devikot	Jaisalmer		26°41'51.75"N/71°11'54.06"E	8.4	2	DC-RJ7, **DC-RJ8**
3.	Ramdevra	Jaisalmer		27°00'38.19"N/71°55'11.02"E	8.5	1	**DC-RJ11**
4.	Mathania	Jodhpur		26°31′47.53″N/72°58′45.81"E	9.0	2	DC-RJ5
5.	JNVU New Campus	Jodhpur		26°14'44.99"N/73°01'17.42"E	8.2	4	**DC-RJ13**, DC-RJ14
6.	Ladnun	Nagaur		27°38'23.50"N/74°23'44.95"E	8.1	2	DC-RJ10
7.	Samdari	Barmer		25°48'51.01"N/72°34'53.13"E	8.1	1	**DC-RJ12**
8.	Lawan	Dausa		26°46'09.09"N/76°12'48.26"E	7.8	4	DC-RJ15, **DC-RJ16**, DC-RJ17
9.	Sirsa	Sirsa	Haryana (HR)	29°32'14.28"N/75°01'57.97"E	8.1	10	**DC-HR18**, DC-HR19, **DC-HR20**, DC-HR21, DC-HR22, DC-HR23
10.	Mansa	Mansa	Punjab (PB)	30°00'03.59"N/75°23'27.70"E	8.0	7	**DC-PB24**, DC-PB25, **DC-PB26**
11.	Semariya	Rewa	Madhya Pradesh (MP)	24°47'38.95"N/81°09'11.48"E	6.8	12	**DC-MP27**, **DC-MP29**, **DC-MP30**, **DC-MP31**, **DC-MP32**
12.	Pondicherry University	Puducherry	Puducherry (PY)	12°01'07.03"N/79°51'12.81"E	7.9	6	DC-PY33, DC-PY34, **DC-PY35**, **DC-PY36**, **DC-PY37**
13.	NEHU Campus, Shillong	East Khasi Hills	Meghalaya (ML)	25°36'49.47"N/91°54'05.17"E	4.9	3	**DC-ML38**, **DC-ML39**, DC-ML40

Note- Strains in bold were identified on the basis of *recA* gene sequence as *Ensifer* (in purple font) and *Bradyrhizobium* (in blue font)

hypochlorite (NaOCl) for 3 min and then washed 6 times in sterile water. The surface sterilized nodules were swabbed onto Yeast Extract Mannitol Agar- Congo red (YEMA-CR) medium to check that sterilization of the nodule surface was 100%, and then crushed into 1–2 drops of sterile distilled water followed by streaking of the white exudate onto YEMA-CR medium. The streaked master plates were incubated at 28 °C for up to 7 days and rhizobia-like colonies (i.e. white or transparent or translucent, circular, smooth margin, raised, EPS producing, mucilaginous or gummy and not taking up the red dye) were picked up for purification. Single colonies were streaked in a four-way pattern on fresh media until pure colonies were obtained (Howieson and Dilworth 2016). Pure cultures were maintained for further analysis, but also preserved as glycerol stocks in -80 °C.

2.3 Phenotypic profiling

Various phenotypic tests were performed on selected strains (identified based on their *recA* gene sequences). The temperature tolerance of the strains was tested by inoculating them onto YEMA and incubating at high temperatures (35 °C, 40 °C, 45 °C and 48 °C). The salt tolerance of the strains was analyzed by streaking them onto YEMA supplemented with a range of salt (NaCl) concentrations: 0.5%, 1%, 2% and 3% (w/v). The pH tolerance of strains was tested by inoculating them onto YEMA adjusted to a range of pH values: 5, 6, 8, 10 and 11 (Sankhla et al. 2017). The acid or alkaline production by strains was tested in YEM broth with Bromo thymol blue (BTB) indicator (Somasegaran and Hoben 1994). Resistance or sensitivity of strains towards seven antibiotics was determined using HiMedia antibiotic discs (single concentration). The metabolic ability of strains to utilize sole carbon (sugar) sources was determined using 21 HiMedia sugar discs in broth containing Andrade pH indicator (Tak et al. 2020).

2.4 Genotypic profiling

Purified bacterial strains were activated in Tryptone yeast (TY) broth in an incubator shaker (120 rpm) at 28 °C for DNA isolation using the method [constituent's phenol, chloroform, STE (Sodium chloride-Tris–EDTA) buffer and TE buffer] of Cheng and Jiang (2006). The purity and concentration of DNA were checked by NanoDrop and DNA of 100 to 1000 ng μl^{-1} was stored at 4 °C for further molecular studies (Tak et al. 2016, 2020), such as DNA fingerprinting, and amplification of housekeeping (*recA*, *glnII*, *atpD* and 16S rRNA) and symbiotic (*nodA* and *nifH*) genes. The RPO1 primer-based Rapid Amplification of Polymorphic DNA (RAPD) profiling was obtained (Richardson et al. 1995) using thermal cycling conditions described in Table S1 for categorizing strains into RAPD genotypes (groups and individual).

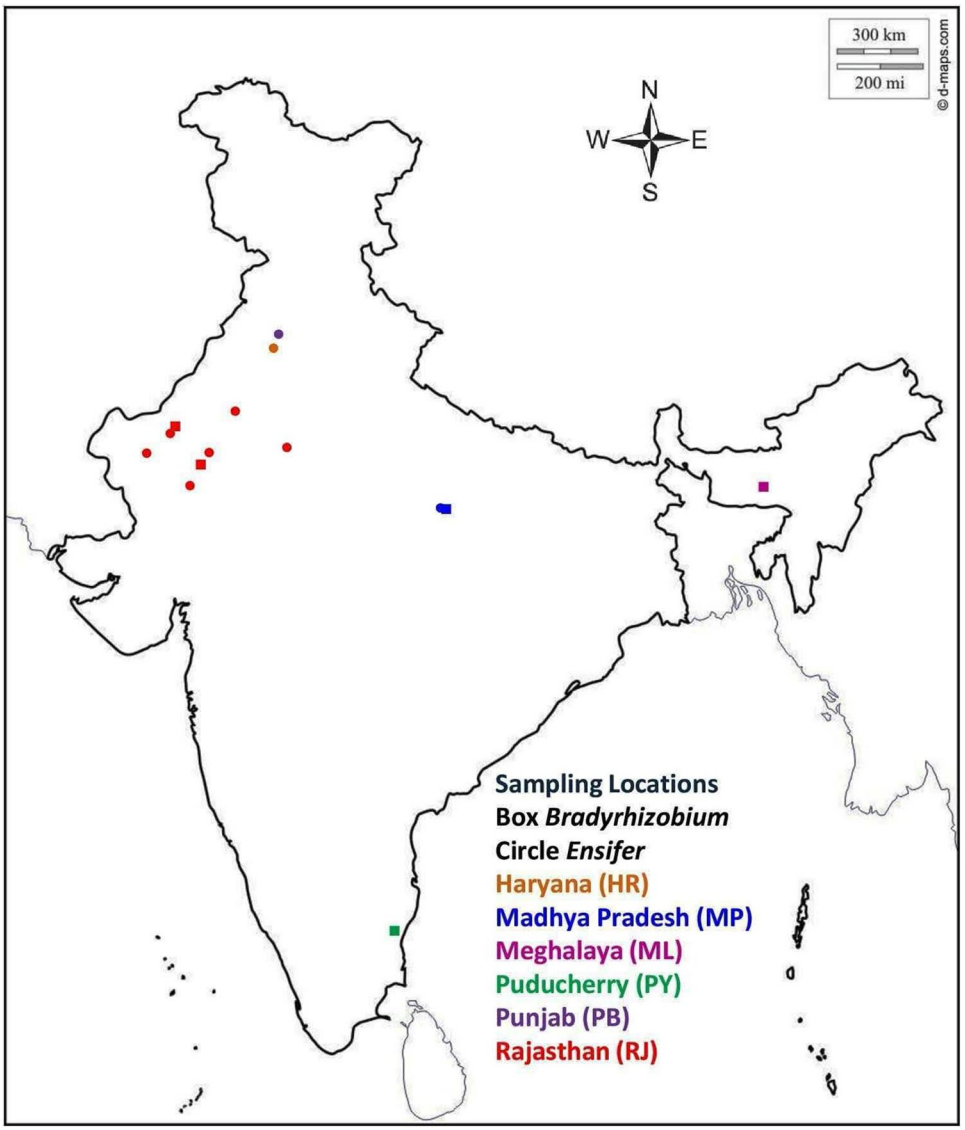

Fig. 1 Map of India showing different soil sampling locations and distribution of *Ensifer* and *Bradyrhizobium* strains isolated from root nodules of host legume *Dichrostachys cinerea*

2.5 Amplification of housekeeping (recA, atpD, glnII and 16S rRNA) and symbiotic (nodA and nifH) genes

For identification of bacterial strains at the molecular level the conserved *recA* (encoding recombination protein A) core gene was successfully amplified using two set of primers, recA6F and recA555R (amplicon size 550 bp), and TSrecAF and TSrecAR (600 bp), for *Ensifer* and *Bradyrhizobium*, respectively (Table S1). The Multi locus Sequence Analysis (MLSA) of selected strains was performed by amplifying additional protein-coding housekeeping genes (*atpD* and *glnII*) and the universal molecular chronometer 16S rRNA gene using different pairs of primers and thermal cycling conditions (Table S1). The symbiosis essential gene *nodA* (encodes N-acyltransferase nodulation protein) was amplified using two sets of primers nodA1 and nodA2 (650 bp), and nodAf.brad and nodAr.brad (550 bp). The *nifH* gene, which encodes nitrogenase reductase protein and is essential for N fixation, was amplified (product size 750 bp) using forward (nifHF) and reverse (nifHI) primers. Details about primers used and thermal cycling conditions used for gene amplification are given in Table S1.

2.6 Sequencing and phylogenetic analysis

Sanger sequencing of purified PCR products of different genes was achieved through an external company (AgriGenome Labs. Pvt. Ltd., Kochi, Kerala, India) that provided results in ABI and FASTA format. Chromatograms and sequences were viewed, edited, and trimmed using Gene Tool lite version 1.0 (Double Twist Inc., Oakland, CA, USA) software. Nucleotide sequences of strains were analyzed for percentage sequence similarity using BLASTn (Nucleotide

Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information). Sequences from the present study and those of closely related, reference and type strains (as per the LPSN list of valid and not validly published type strains of a species in a genus) were downloaded from the NCBI nucleotide in FASTA format, and then aligned using CLUSTALW (Thompson et al. 1994) of MEGA7 (Kumar et al. 2016). Phylogenetic trees (individual or concatenated) were reconstructed in MEGA7 using the Maximum Likelihood method and General Time Reversible (GTR + G + I) model with 1000 bootstrap values.

2.7 Nodulation assay to compare symbiotic efficacy of the rhizobial strains

A selection of genetically characterized *Ensifer* and *Bradyrhizobium* strains were authenticated on their host *D. cinerea* and cross inoculated onto *Vigna radiata*. Surface sterilized seeds were germinated on moist filter paper and transferred (around 5–6 seeds/pot) under aseptic conditions into sterile potting mixture (3:1 soilrite: river sand) in autoclaved plastic pots (Tak et al. 2020). Each seedling was inoculated with 1 ml of bacterial suspension prepared in 1% sucrose. Pots set up in triplicates for each treatment and control were placed in a glasshouse under controlled conditions (28–30 °C) and maintained for 4–6 weeks. Plants were watered with sterile tap water and nourished with N-free nutrient solution (Yates et al. 2004). After 4–6 weeks harvested plants were examined for number of root nodules, shoot fresh weight (g plant^{-1}), shoot dry weight (g plant^{-1}), and compared with un-inoculated control plants either fed with a nutrient solution supplemented with nitrate in the form of KNO_3 (0.1%) or without any added N (Yates et al. 2004; Sankhla et al. 2017; Tak et al. 2020) to determine relative symbiotic efficacy.

3 Results

3.1 Nodulation of Dichrostachys cinerea and nodule anatomy

Dichrostachys cinerea (DC) were found growing profusely in the Botanical garden of the Department of Botany, JNVU, Jodhpur, Rajasthan, India (Fig. S1a). After the monsoon rainfalls these plants grow profusely through root suckers. It has an axillary or extra-axillary spike inflorescence of 2–5 cm long with pink- and yellow-coloured flowers. The apical part of the inflorescence contains yellow hermaphrodite spike flowers, and the lower half part bears pink sterile spike flowers (Fig. S1b). Seeds are glossy, small (4–6 × 3–4.5 mm) and dark brown (Fig. S1c). Two to three months old plants were harvested from pots, and the root system bearing small, round, branched, and indeterminate type of nodules was observed (Fig. S1d). The anatomy and ultrastructure of *D. cinerea* nodules were examined using light microscopy and TEM of sections taken from fixed and resin-embedded nodules originally sampled through trap-experiments (Fig. 2a-d). The nodules were similar to those previously studied on other mimosoid species, such as those in the genera *Mimosa* and *Vachellia* (Gehlot et al. 2013; Sankhla et al. 2017; Choudhary et al. 2020) in that they are indeterminate with single or multilobed meristems (Fig. 2a), and the N_2-fixing zone has both infected and uninfected cells (Fig. 2b). In addition, the invasion zone is relatively small with occasional infection threads (Fig. 2c, d).

3.2 Isolation and purification of potentially symbiotic bacteria from root nodules of Dichrostachys cinerea

A total of 58 bacterial strains were isolated from root nodules of *D. cinerea* grown in rhizospheric as well as non-rhizospheric soils collected from thirteen sites covering five states namely RJ, HR, PB, MP, ML and one union territory PY. The maximum numbers of soil sampling sites were from RJ covering five districts (Jaisalmer, Jodhpur, Nagaur, Barmer and Dausa). The soils from the various sampling sites ranged from alkaline (pH 9) to acidic (pH 4.9). The geographical coordinates of the soil collection sites are listed in Table 1 and marked on a map of India (Fig. 1). The 58 bacterial strains purified from root nodules of *D. cinerea* are listed in Table 1 with their site of isolation. Fast-growing bacterial strains with colony characters such as white, opaque, raised, entire margins, smooth surface, non-mucilaginous, EPS-producing were identified as species of *Ensifer* (10 strains) based on molecular characterization (*recA* gene sequencing and BLASTn). Slow-growing strains with white, opaque, raised, gummy, entire margins, and less mucilaginous colony morphology were identified as *Bradyrhizobium* (10 strains).

3.3 Salt, pH and temperature tolerance of rhizobia

Selected bacterial strains were further characterized phenotypically. Six *Ensifer* strains isolated from RJ and PB showed up to 1% salt (NaCl) tolerance while four *Ensifer* strains isolated from HR and MP survived up to 2% NaCl (Table S2). In contrast, *Bradyrhizobium* strains were comparatively sensitive to salt stress and could not grow in 1% NaCl. Ten *Ensifer* strains showed a pH tolerance range from 6 to 11 while ten *Bradyrhizobium* strains grew in a pH range of 5 to 11 (Table S2). *Bradyrhizobium* strains isolated from PY (DC-PY35, DC-PY36 and DC-PY37) and ML (DC-ML38 and DC-ML39) are tolerant to temperatures from 28 to 35 °C while strains from RJ (DC-RJ11 and DC-RJ13) and MP (DC-MP30, DC-MP31 and DC-MP32) were able

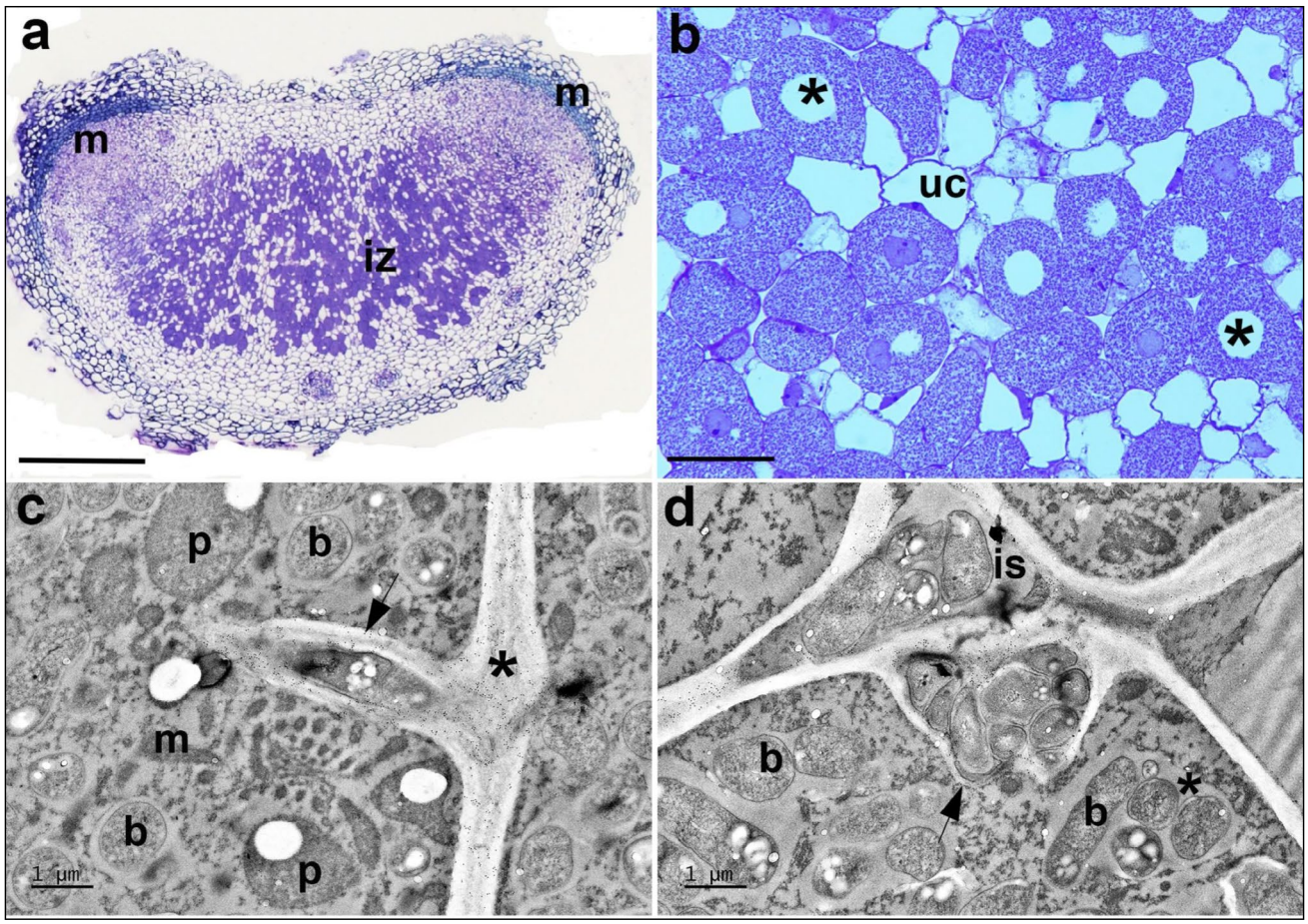

Fig. 2 Structure and ultrastructure of *Dichrostachys cinerea* nodules examined using light microscopy (a, b) and transmission electron microscopy (TEM) (c, d). (a) Longitudinal section (LS) of a multi-lobed nodule with two meristems indicated (m). The N_2-fixing infected zone (iz) consists of dark-stained cells interspersed with non-stained uninfected cells. (b) High magnification view of mature N-fixing cells (*) and uninfected cells (uc) within the infected zone. (c) TEM of an infection thread (IT) entering a host cell in the invasion zone; the IT has originated from the host cell wall between two host cells (*). The wall of the IT (arrow) and the host cell wall (*) are both immunogold labelled with a monoclonal antibody (JIM5) that recognizes a pectin epitope. Note that the host cell receiving the IT is metabolically very active with numerous plastids (p) and mitochondria (m). b = bacteroid. (d) Bacteroids (b) within an infected cell in the N-fixing zone; note that there can be up to four bacteroids per symbiosome (*). A remnant of an IT (arrow) originating from a pocket of bacteria in an intercellular space (is) can also be observed; the walls of this are immunogold labelled strongly with JIM5. Bars = 500 µm (a), 50 µm (b), 1 µm (c, d)

to grow at temperatures from 28 to 48 °C. All the *Ensifer* strains showed tolerance up to 48 °C. In the BTB test all the *Ensifer* strains were acid producers while the *Bradyrhizobium* strains were slightly acidic to slightly alkaline producers plus a few strains showed a neutral response.

3.4 Metabolic profile: carbon utilization and intrinsic antibiotic resistance of rhizobia

Of the six *Ensifer* strains tested (Fig. S2) all utilized Arabinose and Xylose, while *Ensifer* strain DC-MP27 utilized a maximum of 13 sugars. In comparison, the eight tested *Bradyrhizobium* strains utilized fewer sugars except for DC-RJ11. The intrinsic antibiotic resistance (IAR) profiles of the *Ensifer* (10) and *Bradyrhizobium* (10) strains are presented in Table S3. All the tested *Ensifer* strains were resistant to 15 µg Erythromycin except DC-RJ12 that showed a minimum zone of inhibition. The tested *Bradyrhizobium* strains showed resistance against 5 µg Ciprofloxacin and 30 µg Tetracycline except two strains (DC-MP31 and DC-PY36) that showed a minimum zone of inhibition against Tetracycline (Table S3).

3.5 DNA fingerprinting and identification of strains based on recA gene sequences

Of the 58 purified rhizobia 36 strains were selected for genetic fingerprinting using the RPO1 primer based on

colony morphology and site of collection. A total of five genetic groups (Groups I to V) were formed including 16 strains, while the remaining 20 strains had unique banding patterns representing individual genotypes (Table S4). Location dependent banding patterns were observed; Groups I to III consist of strains from RJ, Group IV from HR, while Group V included strains from PB. This grouping indicates that although some strains that are affiliated to a particular geographical area are genetically similar most of them are highly diverse. Based on the RPO1 genetic groups, individual genotypes, and/or their geographical origin (i.e. from sampling sites representing all the different States and Union territories of India in the present study) 20 strains of *D. cinerea* were selected for *recA* gene sequence-based identification and phylogenetic analysis. From the BLASTn results ten were identified as *Ensifer* and ten as *Bradyrhizobium*. NCBI GenBank accession numbers are listed in Table S5.

3.6 Phylogenetic analysis of Ensifer and Bradyrhizobium recA genes

A maximum likelihood phylogenetic tree was constructed using the *recA* gene sequences of ten *Ensifer* (DC-HR18, DC-HR20, DC-MP27, DC-MP29, DC-PB24, DC-PB26, DC-RJ2, DC-RJ8, DC-RJ12 and DC-RJ16) and ten *Bradyrhizobium* (DC-ML38, DC-ML39, DC-MP30, DC-MP31, DC-MP32, DC-PY35, DC-PY36, DC-PY37, DC-RJ11 and DC-RJ13) strains isolated from *D. cinerea* (Fig. 3). The *recA* phylogeny revealed five *Ensifer recA*-types comprising two lineages (EL-I and EL-II) and three clades (EC-I, EC-II and EC-III), the latter two clades (EC-I an EC-II) were placed in the '*Ensifer aridi*' (preferred name and not validly published; Rocha et al. 2020) cluster. This phylogenetic clustering indicated a geographical pattern with strains from soils of a particular state generally grouping together, although a few RJ strains clustered with strains from HR. The strains from RJ, MP, and HR shared similarities with '*E. aridi*' while two PB strains (DC-PB24 and DC-PB26) shared close similarities to *E. kostiensis*.

Similarly, *Bradyrhizobium* strains isolated from different agroclimatic regions presented a clear geographical pattern in their grouping. The seven *Bradyrhizobium recA*-types comprised one clade (BC-I) and six lineages (BL-I to BL-VI) (Fig. 3). Strains belonging to the novel clade BC-I (DC-MP30, DC-MP31, DC-RJ11 and DC-RJ13) and lineage BL1 (DC-MP32) were isolated from arid and semi-arid regions of RJ and MP states, and all were closely related to *B. yuanmingense* in *Bradyrhizobium* Mega Clade-I. Two *Bradyrhizobium* strains (DC-PY35 and DC-PY36) that were isolated from the coastal region of PY were distinct from other *D. cinerea* strains. Strain DC-PY35 diversified from *B. zhanjiangense* whereas DC-PY36 clustered close to it. Another PY strain DC-PY37 occupied a position near *B. ivorense* within *Bradyrhizobium* Mega Clade-II. Two *Bradyrhizobium* strains, DC-ML38 and DC-ML39, both isolated from the wet subtropical climatic conditions and acidic soils of Shillong, ML, were divergent from *B. elkanii* and *B. embrapense*, respectively in Mega Clade-II.

3.7 Multi locus sequence analysis (MLSA) of Ensifer and Bradyrhizobium

In the four (*rrs-glnII-atpD-recA*) gene concatenated phylogeny (Fig. 4) of six selected *Ensifer* strains (DC-HR18, DC-MP27, DC-PB24, DC-RJ2, DC-RJ12 and DC-RJ16) five MLSA types (MLSA T-I to V) were formed, and congruence was observed in the phylogenetic positioning of the strains *vis a vis* the individual housekeeping gene based phylogenies. From the five MLSA types three appear to be novel and are potentially new species of *Ensifer*. Strains DC-RJ2 and DC-HR18 from (T-I) were identical to '*E. aridi*' LMR001 (isolated from *Vachellia gummifera*, Morocco; Le Quéré et al. 2017; Rocha et al. 2020) whereas the strain DC-MP27 (T-II) formed a lineage close to a group of Indian '*E. aridi*'-type strains. Both in the individual and concatenated core gene phylogenies strain DC-PB24 (T-III) clustered close to *E. kostiensis* (isolated from *Senegalia senegal*, Sudan) and strains LL-HR123 and LL-PB121 isolated from *L. leucocephala* grown in HR and PB soils, respectively, by Chouhan et al. (2022). Strain DC-RJ12 (T-IV) shared close similarity with *Ensifer* sp. CPTN45 isolated from root nodules of *Chamaecrista pumila* grown in soils from Tamil Nadu, India (Rathi et al. 2018), while strain DC-RJ16 (T-V) formed a novel lineage and was divergent to *Ensifer* sp. LL-RJ68 (isolated from *L. leucocephala*, RJ, India).

The maximum likelihood concatenated four (*recA-glnII-atpD-rrs*) gene phylogeny of six (DC-ML38, DC-ML39, DC-MP30, DC-PY35, DC-PY37 and DC-RJ13) *Bradyrhizobium* strains resembled the individual housekeeping gene phylogenies. However, the *Bradyrhizobium* MLSA types (T-Ia, T-Ib, T-II, T-III, T-IV and T-V) formed in the concatenated phylogenetic tree (Fig. 5) gave better taxonomic resolution revealing the apparent novelty of the strains and hinting at their potential to belong to new species. MLSA types T-Ia (DC-RJ13) and T-Ib (DC-MP30) formed a distinct novel clade with *C. pumila* symbionts from soils of RJ and with strain LL-MP86 from *L. leucocephala* (MP, India). One PY strain, DC-PY35, clustered in Mega Clade-I close to *B. zhanjiangense* while strain DC-PY37 clustered in Mega Clade-II close to *B. ivorense*. The Shillong *Bradyrhizobium* strains DC-ML38 and DC-ML39 formed novel lineages in Mega Clade-II and were closely related to *B. embrapense* and strain EHNEHU6 isolated from root nodules of *Eriosema chinense* (ML, India).

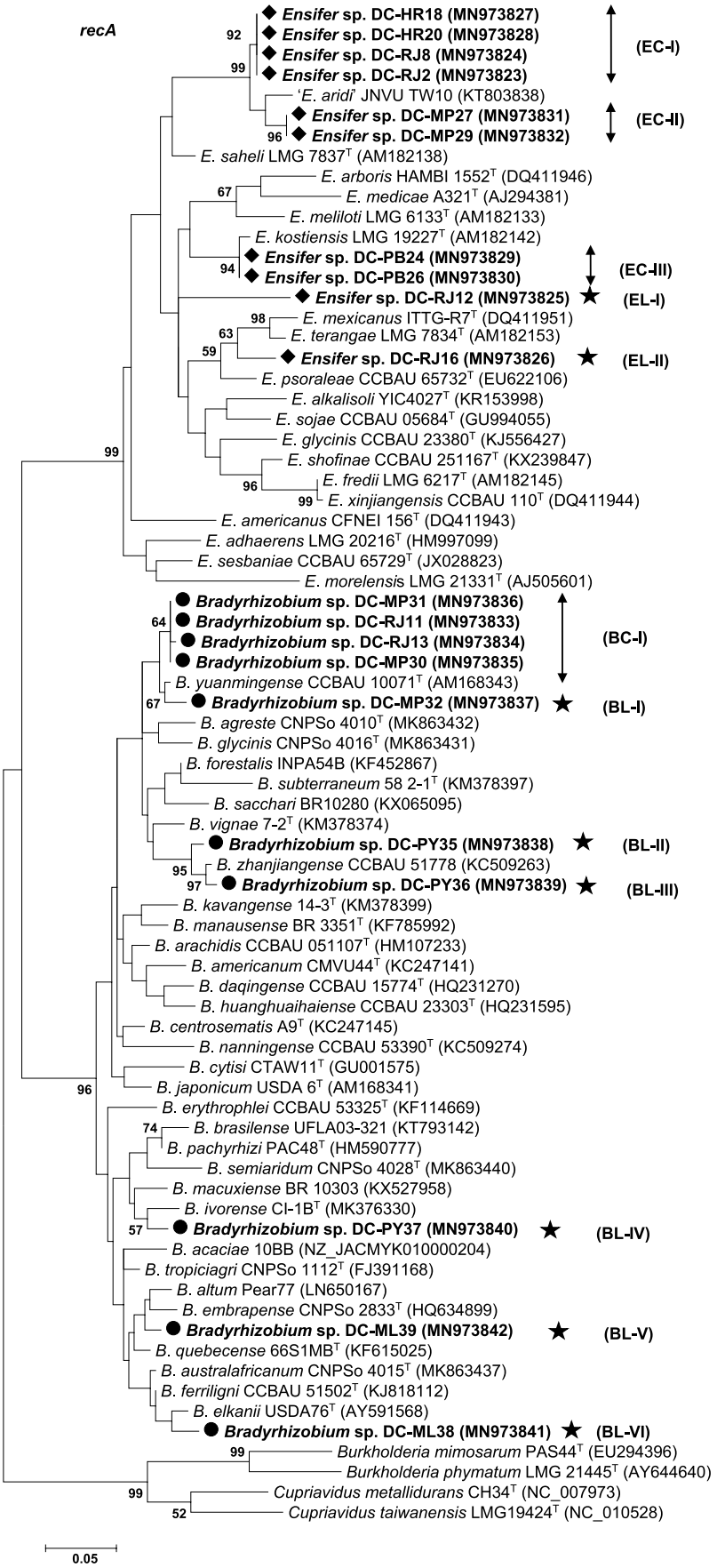

Fig. 3 Maximum Likelihood phylogenetic tree of ten *Ensifer* and ten *Bradyrhizobium* strains isolated from host *Dichrostachys cinerea* with type strains reconstructed using *recA* gene sequences. Bootstrap values calculated for 1,000 replications and above 50% value indicated at internodes. The scale bar represents 5% nucleotide substitution per site. Abbreviations: *B*., *Bradyrhizobium*; *E*., *Ensifer*; BC, *Bradyrhizobium* clade; EC, *Ensifer* clade; BL, *Bradyrhizobium* lineage; EL, *Ensifer* lineage and superscripted T, Type strain. (GenBank accession numbers are in parenthesis)

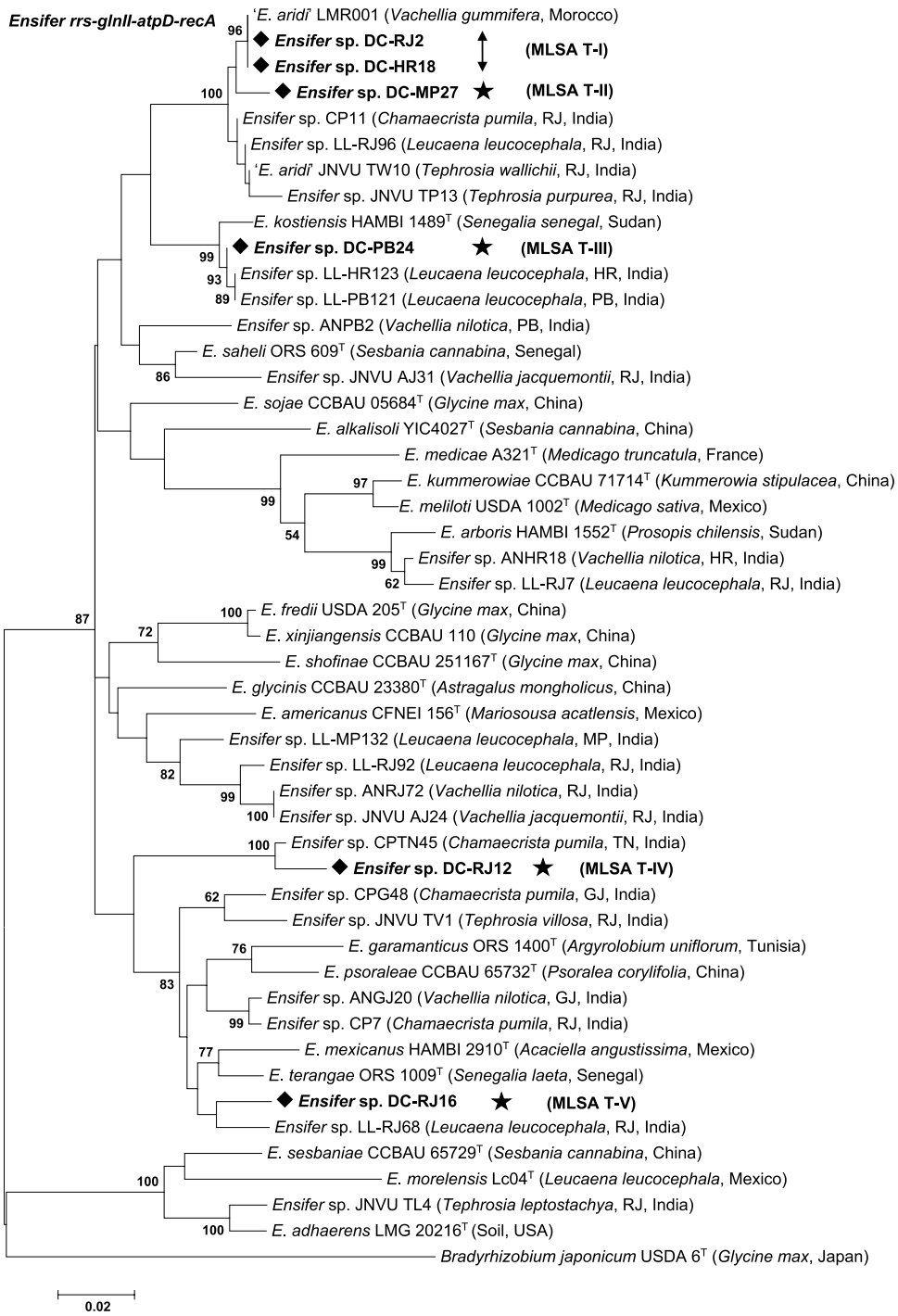

Fig. 4 Maximum Likelihood phylogenetic tree of six *Ensifer* strains isolated from host *Dichrostachys cinerea* with type strains and other *Ensifer* strains isolated from Indian soil reconstructed using concatenated *rrs-glnII-atpD-recA* gene sequences. Bootstrap values calculated for 1,000 replications and above 50% value indicated at internodes. The scale bar represents to 2% nucleotide substitution per site. Following abbreviations represents to: *E.*, *Ensifer*; MLSA, multi locus sequence analysis; T, Type and superscripted T, Type strain

3.8 Symbiosis essential gene (nodA and nifH) phylogenies of Ensifer and Bradyrhizobium

A phylogenetic analysis was conducted on the symbiosis essential genes *nodA* and *nifH* of ten *Ensifer* and ten *Bradyrhizobium* strains isolated from *D. cinerea* in India. The ten *Ensifer* strains were resolved into four *nodA* types (T-i to T-iv) (Fig. 6). Two RJ strains (DC-RJ2 and DC-RJ8) (*nodA* type T-ia and b) grouped with '*E. aridi*'-type strains isolated in India from root nodules of *Tephrosia* spp., *C. pumila* and *L. leucocephala*. One RJ (DC-RJ16) and two HR (DC-HR18 and DC-HR20) strains grouped to form the variant *nodA* type T-ii, while *Ensifer* strains from PB (DC-PB24 and DC-PB26) and MP (DC-MP27 and DC-MP29) clustered together with 100% similarity to *Ensifer* sp. CPG48 (*C. pumila*, Gujarat, India) in the *nodA* type T-iii. Three *nodA* types (T-i to T-iii) can be considered as mega clades of local s*ym* genes which

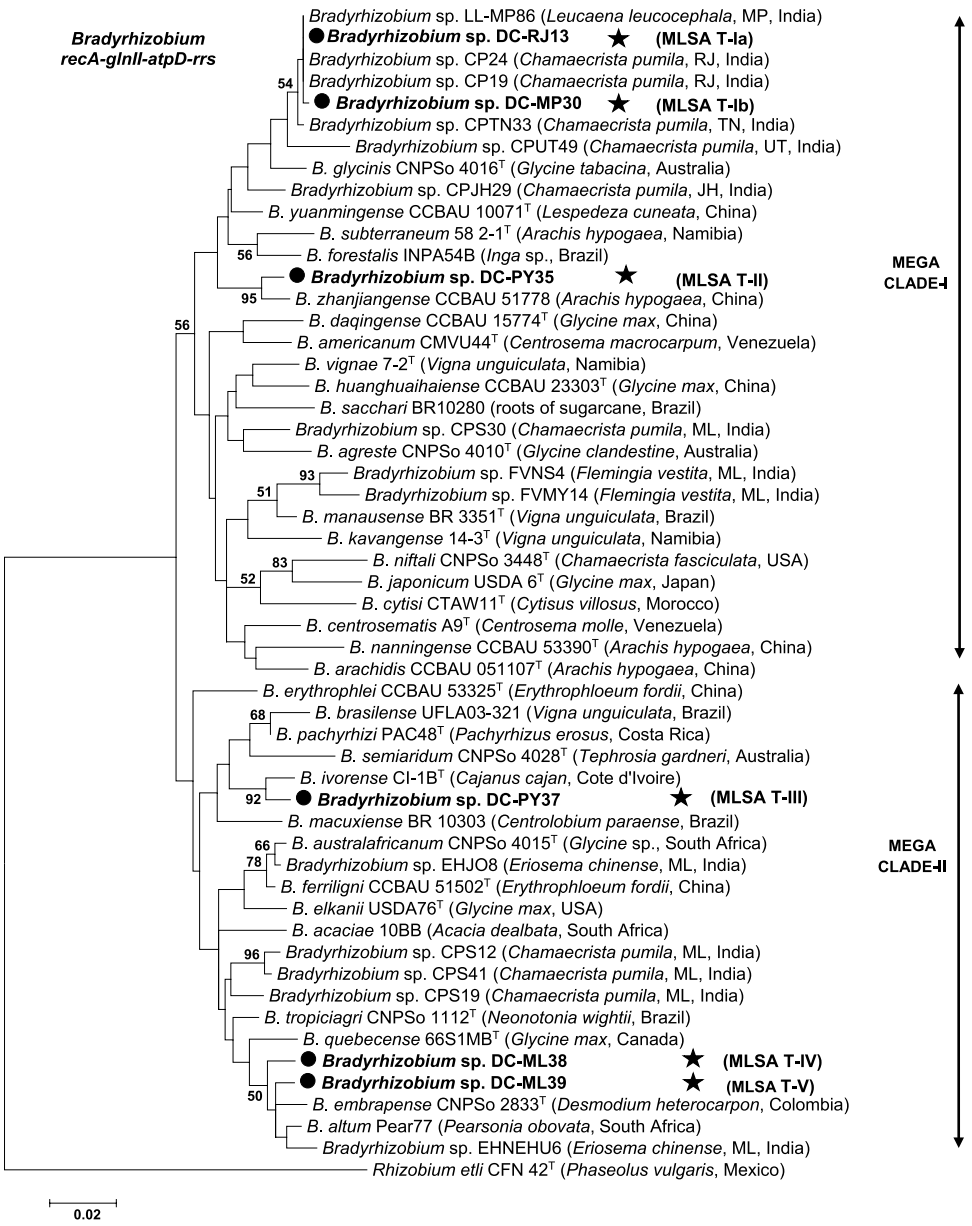

Fig. 5 Maximum Likelihood phylogenetic tree of six *Bradyrhizobium* strains isolated from host *Dichrostachys cinerea* with type strains and other *Bradyrhizobium* strains isolated from India reconstructed using concatenated *recA-glnII-atpD-rrs* gene sequences. Bootstrap values calculated for 1,000 replications and above 50% value indicated at internodes. The scale bar represents to 2% nucleotide substitution per site. Following abbreviations represents to: *B*., *Bradyrhizobium*; MLSA, multi locus sequence analysis; T, Type and superscripted T, Type strain

are harbored by a large group of local legumes suggesting these genes are more promiscuous and not specific to any particular host. Only a single strain, DC-RJ12 (*nodA* type T-iv), from RJ was positioned within the major clade of Indian mimosoid-*nodA* types closely related to *E. arboris*, while the remaining *Ensifer* strains harbored novel *nodA* types clustering in a mega clade positioned close to *E. fredii*. Strains such as DC-HR18, DC-HR20, DC-MP27, DC-MP29, DC-RJ2 and DC-RJ8 shared close similarity to '*E. aridi*' in their *nodA* and *recA* gene phylogenies. In the *nifH* phylogeny (Fig. S3) ten *Ensifer* strains showed a similar pattern of phylogenetic divergence from type strains as observed in the *nodA* phylogeny. Strains from RJ (DC-RJ2, DC-RJ8 and DC-RJ16) and HR (DC-HR18 and DC-HR20) clustered into a single clade while strains of PB (DC-PB24 and DC-PB26) and MP (DC-MP27 and DC-MP29) separated into two locational sub-types in the *nifH* phylogeny (Fig. S3). Strain DC-RJ12 grouped close to *Ensifer* sp. LL-RJ7 (from *L. leucocephala*) and *Ensifer* strains isolated from *V. nilotica* in HR and Gujarat (Choudhary et al. 2020).

In the *nodA* phylogeny the ten selected *Bradyrhizobium* strains dispersed to form six *nodA* types (T-i to T-vi) (Fig. 7). Locational clustering was observed in the *nodA* phylogenetic diversity i.e. two RJ strains were closely related to *B. yuanmingense* and resolved into *nodA* type T-ia (DC-RJ11) and T-ib (DC-RJ13), while strains from PY (DC-PY35 and DC-PY36) and MP (DC-MP30, DC-MP31 and DC-MP32) resolved into *nodA* type T-ii and T-iii, respectively, forming novel clades divergent

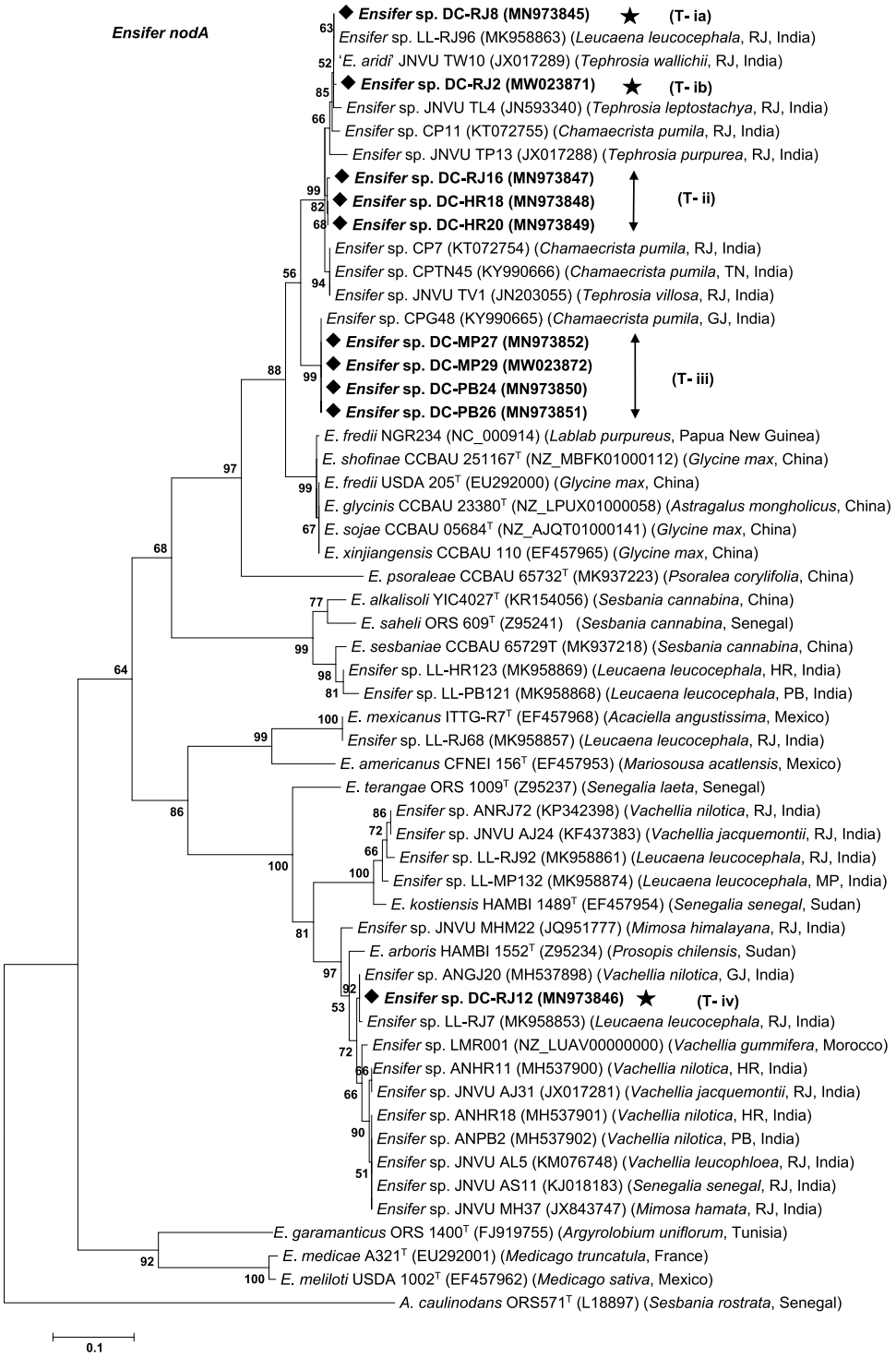

Fig. 6 Maximum Likelihood phylogenetic tree of ten *Ensifer* strains isolated from host *Dichrostachys cinerea* with type strains and other *Ensifer* strains isolated from Indian soil reconstructed using *nodA* gene sequences. Bootstrap values calculated for 1,000 replications and above 50% value indicated at internodes. The scale bar represents to 10% nucleotide substitution per site. Following abbreviations represents to: *A*., *Azorhizobium*; *E*., *Ensifer*; T, Type and superscripted T, Type strain. (GenBank accession numbers are in parenthesis)

to *B. agreste* isolated from *Glycine clandestina* in Australia (Klepa et al. 2021). These two novel clades showed incongruence in their phylogenetic positions in housekeeping and *sym* gene phylogenies while the phylogenetic position of strain DC-PY37 (T-iv) showed congruence. Strain DC-ML38 (T-v) was divergent from *B. elkanii* and strain DC-ML39 (T-vi) clustered with previously reported strains from ML, India. The *nifH* phylogeny of the ten *Bradyrhizobium* strains was similar to that of *nodA* (Fig. S4). *Ensifer* and *Bradyrhizobium* strains from the present study harbored *sym* genes overlapping with other local symbionts reported from India.

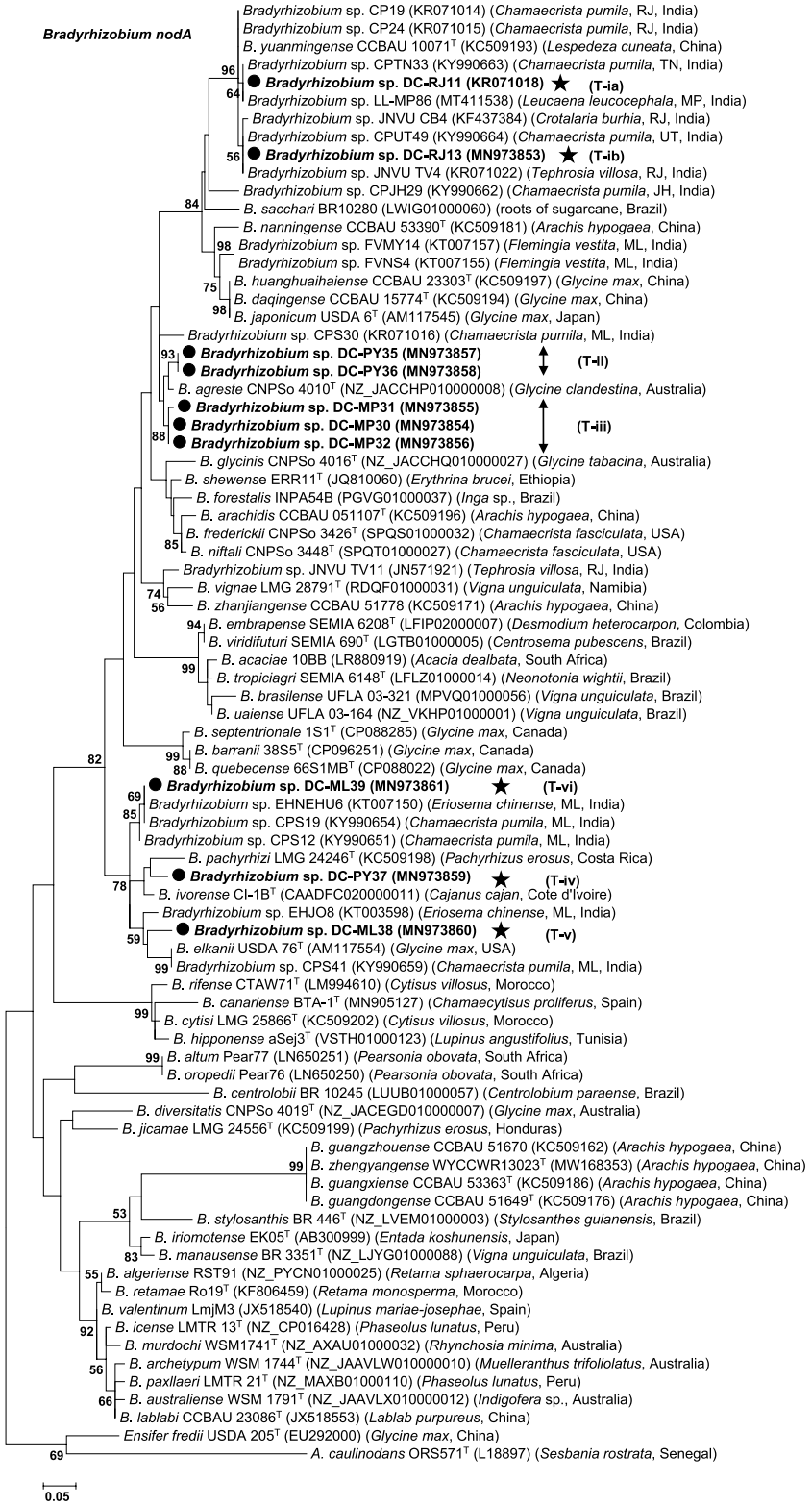

Fig. 7 Maximum Likelihood phylogenetic tree of ten *Bradyrhizobium* strains isolated from host *Dichrostachys cinerea* with type strains and other *Bradyrhizobium* strains isolated from Indian soil reconstructed using *nodA* gene sequences. Bootstrap values calculated for 1,000 replications and above 50% value indicated at internodes. The scale bar represents to 5% nucleotide substitution per site. Following abbreviations represents to: *A*., *Azorhizobium*; *B.*, *Bradyrhizobium*; T, Type and superscripted T, Type strain. (GenBank accession numbers are in parenthesis)

3.9 Symbiotic efficacy of Ensifer and Bradyrhizobium

Strains belonging to different MLSA and *nodA* types of *Ensifer* (DC-HR18, DC-MP27, DC-PB24, DC-RJ2 and DC-RJ16) and *Bradyrhizobium* (DC-ML38, DC-ML39, DC-MP30, DC-PY35, DC-PY37 and DC-RJ13) were cross inoculated onto an important crop legume, *Vigna radiata*, to determine their symbiotic efficacy (Fig. 8a-c). All tested *Ensifer* and *Bradyrhizobium* strains except

DC-PY37 nodulated *V. radiata*. Overall, the *Ensifer* strains formed more root nodules per plant than the *Bradyrhizobium* strains, with *Ensifer* strain DC-HR18 forming the highest number of nodules (Fig. 8a). Shoot fresh weight (Fig. 8b) and shoot dry weight (Fig. 8c) of plants inoculated with *Ensifer* and *Bradyrhizobium* strains was higher in comparison to un-inoculated N -minus control plants. A few plants inoculated with *Ensifer* strains (DC-HR18, DC-MP27, DC-RJ2 and DC-RJ16) and *Bradyrhizobium* (DC-PY35 and DC-RJ13) even performed better than the N + control. Symbiotically, the best-performing strain on *V. radiata* was *Ensifer* sp. DC-RJ16.

4 Discussion

4.1 Nodulation of Dichrostachys cinerea in different soils and selection of rhizobia

Gehlot et al. (2012) reported nodulation in the perennial shrub *D. cinerea* and Chouhan et al. (2020) later observed its nodulation with slow-growing *Bradyrhizobium* and fast-growing *Ensifer* strains in arid and semi-arid regions of Rajasthan, India. In the present study we have greatly expanded these aforementioned studies, and have also obtained information about the anatomy of *D. cinerea*

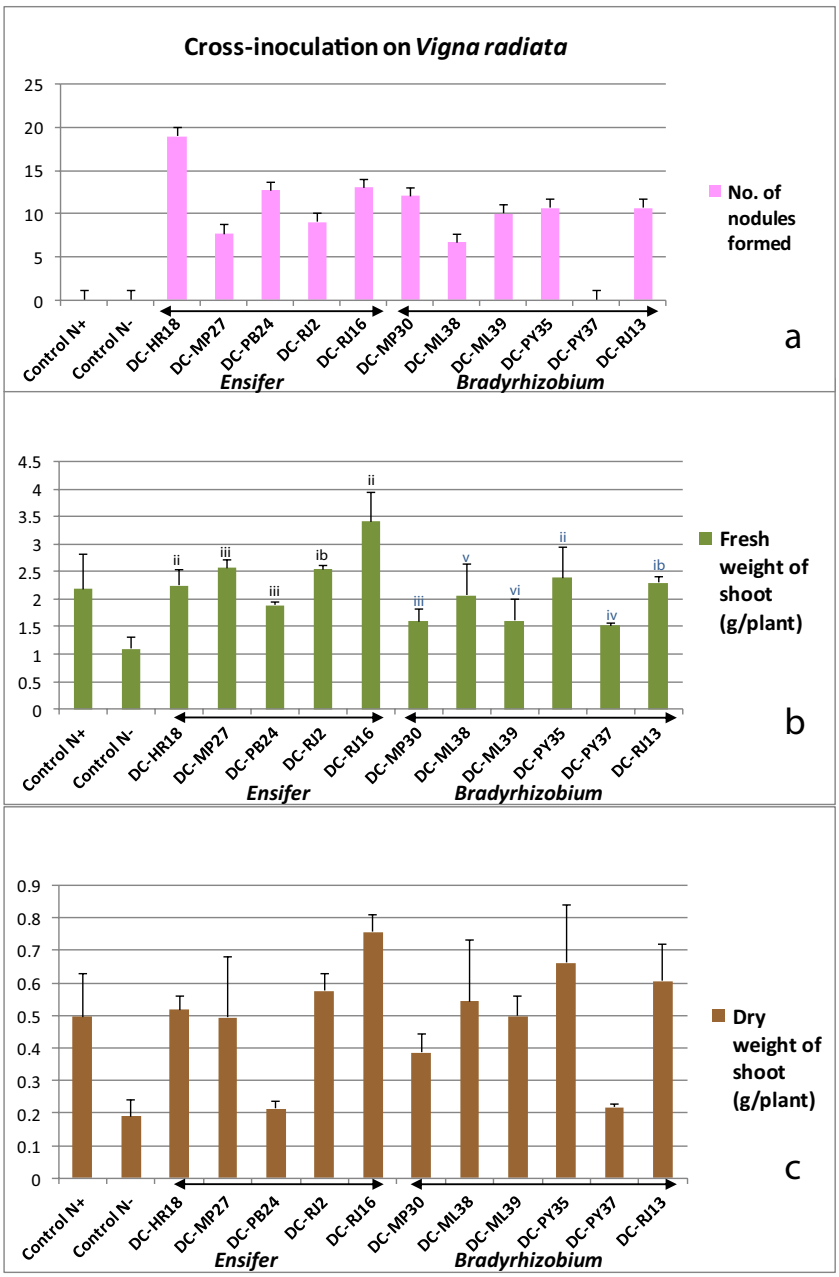

Fig. 8 Average number of nodules (a), fresh weight of shoot (b) and dry weight of shoot (c) per plant of *Vigna radiata* inoculated with strains of *Ensifer* and *Bradyrhizobium* in comparison to un-inoculated N + and N- control plants. (*nodA* types are indicated at top of standard deviation bar for parameter fresh weight of shoot)

nodules. Nodules formed on *D. cinerea* were anatomically and ultra-structurally similar to those reported previously on Mimosoid legumes in general (de Faria et al. 2022). This study thus reinforces the notion that Mimosoid legumes have evolved a particular nodule type that is highly conserved within this clade, but also different from all other legume nodule types, including those within their parent subfamily Caesalpinioideae (Sprent et al. 2017). Indeed, it is now considered likely that the high incidence of nodulation in the Mimosoid clade compared to the largely non-nodulated non-mimosoid Caesalpinioideae is due to their rejection of the cell wall-bound "fixation thread" type of bacteroid that is present in the latter, and their adoption of the more intimate and efficient membrane-bound symbiosome that is more redolent of "advanced" SYM-type nodules in the subfamily Papilionoideae (de Faria et al. 2022).

To identify the root nodule microsymbionts associated with *D. cinerea*, a wide sampling from the state of RJ was performed together with more limited sampling from other states and union territories of India with varied agroclimatic conditions. In addition to analysing the phylogenetic diversity of its microsymbionts, the symbiotic efficacy and the compatibility of wild rhizobia on the crop legume *V. radiata* was also investigated. The pH of soils collected from different sampling sites ranged from alkaline to neutral to acidic, but *D. cinerea* nodulated in all the soils used for the trap experiment. In contrary to this Mimosoids such as *V. jacquemontii* (Sankhla et al. 2017), *V. nilotica* (Choudhary et al. 2020) and *L. leucocephala* (Chouhan et al. 2022) failed to nodulate in the acidic soils of ML suggesting that in addition to edaphic and geographical factors (Pires et al. 2018; Rathi et al. 2018), host preferences also play key roles in establishing successful symbiosis. A total of 58 bacterial (both fast- and slow-growing) strains were isolated and purified from root nodules of *D. cinerea*. DNA fingerprinting of 36 selected bacteria showed considerable genetic diversity in the banding patterns with some location-specific genetic groups suggesting biogeographical factors are least partly responsible (Sprent et al. 2017). *Dichrostachys cinerea* nodulated with strains of both *Ensifer* and *Bradyrhizobium* in the alkaline (RJ, HR, PB, PY) and neutral (MP) soils; while in acidic soils (ML) only *Bradyrhizobium* microsymbionts were isolated. Such soil pH-based distribution of *Ensifer* and *Bradyrhizobium* are similar to previous reports that suggests *Ensifer* as a predominant root nodule microsymbiont in alkaline soils while *Bradyrhizobium* predominates in acidic soils (Gehlot et al. 2012; Tak et al. 2016; Sankhla et al. 2017; Ojha et al. 2017; Rathi et al. 2018; Choudhary et al. 2020; Jorrin et al. 2021; Chouhan et al. 2022). Phenotypic variation, such as distinctive carbon utilization and IAR patterns was also observed among the tested *Bradyrhizobium* and *Ensifer* strains, with a few strains that could tolerate high salt concentrations (up to 3%) and high temperature (up to 48 °C) as reported earlier (Zhang et al. 1991; Tak et al. 2016; Sankhla et al. 2015, 2017; Rathi et al. 2017; Choudhary et al. 2017, 2018, 2020; Gaur et al. 2018; Chouhan et al. 2020, 2022).

4.2 Phylogenetic diversity and mosaicism of core and sym genes in D. cinerea-nodulating Ensifer and Bradyrhizobium strains

In comparison to 16S rRNA, phylogenies based on the protein coding *recA* gene have better resolution (Tak et al. 2016; Sankhla et al. 2017; Rathi et al. 2018; Chouhan et al. 2022). Accordingly, 20 bacterial strains isolated from different sampling sites were identified as species of *Ensifer* and *Bradyrhizobium* based on their *recA* genes. Of the five MLSA types formed in concatenated gene phylogeny three are novel types divergent from the described type strains. Based on multiple core gene loci *Ensifer* strains DC-RJ2 and DC-HR18 shared similarity with '*E. aridi*' (strain LMR001 isolated from *V. gummifera* in North Africa Le Quéré et al. 2017), but differed in *sym* loci (*nodA* and *nifH*) suggesting these strains have typically Old World core genomes, but have evolved in terms of plasmid-borne loci. The strains DC-MP27, DC-PB24, DC-RJ12 and DC-RJ16 showed typical incongruence in core and *sym* loci. Based on core and *nodA* genes majority of *D. cinerea- Ensifer* strains were closely related with the symbionts of *C. pumila* and *L. leucocephala* (Rathi et al. 2018; Chouhan et al. 2022). Diverse *Ensifer* strains have been identified from *D. cinerea* trapped in PB and HR soils. From these same sampling sites [Mansa (PB) and Sirsa (HR)] *Ensifer* strains were trapped in root nodules of *V. nilotica* (Choudhary et al. 2020) and *L. leucocephala* (Chouhan et al. 2022) but the *Ensifer* genotypes isolated from these three host plants are divergent from each other. Symbiotic preferences for *Ensifer* strains by members of the Caesalpinioideae (including the Mimosoids), and the Papilionoideae have evolved differently. Some mimosoids such as *L. leucocephala* (Chouhan et al. 2022) and *D. cinerea* (this study) are able to establish symbiosis both with Papilionoid-derived *Ensifer* and Mimosoid-derived *Ensifer* types (Tak et al. 2016; Sankhla et al. 2017; Rathi et al. 2018; Tak and Gehlot 2019; Choudhary et al. 2020; Chouhan et al. 2022). *Ensifer* strains from the non-Mimosoid Caesalpinioideae species *C. pumila* shared similarity only with Papilionoid-derived *Ensifer* and did not associate with Mimosoid-derived *Ensifer* strains (Rathi et al. 2018) highlighting the complexity of host factors involved in the legume-rhizobia symbiosis. It is intriguing question if legume host have evolved to pair with multiple types of microsymbionts or if the microsymbionts have evolved to infect multiple host plants and hence define promiscuity. As reported by multiple studies (Sankhla et al. 2017; Rathi et al. 2018; Choudhary et al. 2020; Chouhan et al. 2022) the

present study on *D. cinerea-Ensifer* reinforces the notion that *Ensifer* is the dominant root nodule microsymbiont of native and invasive legumes in alkaline soils of India, and that the enormous genetic diversity of *Ensifer* has been created through horizontal gene transfer (HGT) resulting in several mosaic combinations of core and *sym* genes (Tak et al. 2016; Sankhla et al. 2017; Andrews et al. 2018; Chouhan et al. 2022).

The concatenated phylogeny of *Bradyrhizobium* provided clarity in the taxonomic positions of strains that were dispersed in *Bradyrhizobium* Mega Clade-I and II forming several novel MLSA types. Strains DC-RJ13 and DC-MP30 were divergent from *B. yuanmingense* based on their core genomes but shared similarity with strains reported from *C. pumila* and *L. leucocephala* (Rathi et al. 2018; Chouhan et al. 2022). Strains with such a genetic make-up are predominant in the alkaline soils of arid and semi-arid regions of Thar Desert (RJ) and other states of India, and even in Pakistan (Appunu et al. 2009; Gehlot et al. 2012; Choudhary et al. 2017; Rathi et al. 2017, 2018; Sankhla et al. 2018; Tak and Gehlot 2019; Chouhan et al. 2020, 2022; Jorrin et al. 2021; Hakim et al. 2021). The *D. cinerea*-nodulating *Bradyrhizobium* MLSA types Ia and Ib harbored completely divergent *nodA* genes whereby DC-RJ13 retained its position with other *Bradyrhizobium* strains reported from alkaline RJ soils, but strain DC-MP30 clustered with other MP strains to form a novel *sym*-type clade. This is the first incidence in which we have found *B. yuanmingense*-type strains showing incongruence in their core and *sym* gene phylogenies. Strains DC-PY35 and DC-PY37 were trapped by *D. cinerea* grown in PY soils collected from the rhizosphere of *L. leucocephala*. Both strains (DC-PY35 and DC-PY37) are symbionts that are apparently specific to *D. cinerea* on the basis of their core genes and *nodA* loci. Interestingly, the exotic legume *L. leucocephala* was nodulated only by strains of *Ensifer* in these same soils and did not select *Bradyrhizobium* (Chouhan et al. 2022). Moreover, at the pan-India level sampling/rhizobia trap experiments with *L. leucocephala* the *Bradyrhizobium* strains were trapped only from MP soils while in the case of *D. cinerea Bradyrhizobium* strains were trapped from sampling sites covering RJ, PY (alkaline), MP (neutral) and ML (acidic) soils. Therefore, the host genotype clearly plays a role in the selection of rhizobia from the same soil. Fields et al. (2023) in a recent study on clover reported the influence of host selection and local growth conditions on diversity and composition of *Rhizobium* within clover nodules. Strains DC-ML38 and DC-ML39 isolated from acidic soils of ML formed novel lineages in Mega Clade-II. Remarkably *L. leucocephala* failed to nodulate in acidic ML soils collected from NEHU Campus, Shillong (Chouhan et al. 2022) but *D. cinerea* effectively picked up *Bradyrhizobium* suggesting a more elaborated promiscuity of this invasive mimosoid. As reported in Rathi et al. (2018) our results also suggests that a significant diversity of *Bradyrhizobium* has evolved in the acidic soils of ML which has a large pool of novel *Bradyrhizobium* strains carrying a mosaic of novel combinations of core and *sym* genes.

4.3 Symbiotically efficient Ensifer and Bradyrhizobium harbor diverse sym genes

Ten *Ensifer* strains phylogenetically resolved into four *nodA* types of which three types (T-i, ii, iii) were identical or closely related to the Indian '*E. aridi*'-type of strains but were divergent to the African '*E. aridi*' strain LMR001 (Tak et al. 2016; Rathi et al. 2018; Tak and Gehlot 2019; Rocha et al. 2020; Chouhan et al. 2022) while one type (T-iv) grouped within an exclusively Indian mimosoid-*nodA* clade. Incongruence was observed in the phylogenetic positioning of *D. cinerea-Ensifer* strains in core and *nodA* phylogenies with a few genetically diverse strains harboring a common *nodA*. Most *Ensifer* strains from Indian mimosoid legumes harbor novel *nodA* genes closely related to *E. arboris* and some harbor an *E. kostiensis*-type (Sankhla et al. 2017; Choudhary et al. 2017, 2018, 2020; Chouhan et al. 2022). Remarkably, the '*E. aridi*' *nodA*-type has not been previously encountered in *Ensifer* symbionts of native Mimosoid legumes, including species of *Mimosa* (Gehlot et al. 2013), *Vachellia* (Sankhla et al. 2017; Choudhary et al. 2017, 2020) and *Senegalia* (Choudhary et al. 2018), but *Ensifer* symbionts of the two exotic mimosoids (*D. cinerea* and *L. leucocephala*) and native *Prosopis cineraria* (Gehlot et al. 2016) have acquired this dominant and widely distributed *nodA* gene. The exotic legumes *D. cinerea* and *L. leucocephala* are nodulated by both (i) *Ensifer* with Mimosoid-*nodA* type and (ii) *Ensifer* strains that nodulate several Papilionoids (*Alysicarpus vaginalis*, *Rhynchosia aurea*, *Crotalaria burhia* and *Tephrosia* spp.) harboring novel Papilionoid-*nodA* genotypes (Rathi et al. 2017; Tak et al. 2016; Sankhla et al. 2018; Tak and Gehlot 2019). *Bradyrhizobium* strains from PY (DC-PY35 and DC-PY36) and MP (DC-MP30, DC-MP31 and DC-MP32) clustered to form novel clades divergent to *B. agreste* (isolated from *Glycine clandestina*, Australia) (Klepa et al. 2021). A similar clustering was observed in strains originating from acidic soils of ML (Ojha et al. 2017; Rathi et al. 2018). Strains DC-RJ11 and DC-RJ13 from alkaline soils of RJ were divergent to *B. yuanmingense* in both core and *sym* gene phylogenies, and shared close similarities with many strains reported from several Papilionoids (*A. vaginalis*, *R. aurea*, *C. burhia* and *Tephrosia* spp.), Caesalpinioideae-Mimosoids (*V. leucophloea* and *L. leucocephala*) and non-Mimosoid Caesalpinioideae (*C. pumila*) from different states (RJ,

MP, Tamil Nadu, Uttarakhand) of India (Choudhary et al. 2017; Rathi et al. 2017, 2018; Sankhla et al. 2018; Tak and Gehlot 2019; Chouhan et al. 2022).

The symbiotic performance of *Ensifer* strains harboring different *nodA* types on the crop legume *V. radiata* was comparable as all nodulated and improved the biomass of inoculated plants in comparison to N-control plants, as did the *Bradyrhizobium* strains. Similar results have been reported by Tak et al. (2013, 2016), Gehlot et al. (2016), Le Quéré et al. (2017) and Rathi et al. (2018) whereby *Ensifer* strains harboring the '*E. aridi*'-type of *nodA* effectively nodulated *V. radiata* whereas *Ensifer* strains harboring the "standard" Indian mimosoid type of *nodA* fail to nodulate *Vigna* spp. as reported by Sankhla et al. (2017), Choudhary et al. (2017, 2018, 2020) and Chouhan et al. (2022), suggesting different host preferences based on the rhizobial *sym* type.

5 Conclusion

Dichrostachys cinerea is native to Africa, but in India it grows well and is nodulated in alkaline and acidic soils. Diverse strains of *Ensifer* and *Bradyrhizobium* occupy its root nodules in equal proportion depending on the local soil type. The MLSA of strains from *D. cinerea* is the first documentation of the phylogenetic diversity of its microsymbionts from India and elsewhere. The preferences in terms of selecting microsymbionts by *D. cinerea* is similar to that previously reported in India for native and exotic Caesalpinioideae, such as the invasive *L. leucocephala* (Chouhan et al. 2022) and the native *C. pumila* (Rathi et al. 2018) in that it is very promiscuous effectively nodulating with diverse strains of *Ensifer* (mainly Old World) and *Bradyrhizobium* (from both Mega clades). The symbiotaxonomy of *Ensifer* strains also differed as they cross-nodulated the crop *V. radiata*, which previously reported Indian mimosoid-nodulating *Ensifer* strains failed to achieve. Strains with such broad host range expanding from Mimosoids to legume crops are ideal candidates for inoculum preparation to improve soil fertility. Moreover, since *D. cinerea* nodulates and fix N in association with a broad diversity of rhizobia in alkaline soils and acidic soils of India, provided proper controls of its invasiveness are implemented, its cultivation can be advocated to increase soil fertility in both kinds of soil environment.

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Declarations

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

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