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ORIGINAL ARTICLES

Numerical analysis of phenotypic properties, genomic fingerprinting, and multilocus sequence analysis of *Bradyrhizobium* strains isolated from root nodules of *Lembotropis nigricans* of the tribe Genisteae

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Abstract

Purpose The aim of this study was to estimate the level of genomic and phenotypic diversity as well as the genus and species position of bacterial strains isolated from root nodules of *Lembotropis nigricans* (family Fabaceae).

Methods The genomic diversity of studied *L. nigricans* nodule symbionts was examined by using BOX-PCR and AFLP (amplified fragment length polymorphism) fingerprinting techniques. To assign bacteria to the genus, numerical analysis of phenotypic features and comparative analysis of 16S rDNA sequences were performed. The comparative analysis of combined *atpD*, *dnaK*, *gyrB*, and *rpoB* gene sequences (multilocus sequence analysis, MLSA) was used to determine the most closely related species to the studied bacteria.

Results Both BOX-PCR and AFLP techniques revealed a high level of genomic heterogeneity of *L. nigricans* nodulators. Among 33 studied bacteria, 32 genotypes were delineated by the AFLP method and 27 genotypes were identified by the BOX-PCR fingerprinting. The numerical analysis of 86 phenotypic characteristics of *L. nigricans* nodule isolates and reference rhizobia showed that studied bacteria belong to the genus *Bradyrhizobium*. Affiliation of *L. nigricans* nodule isolates to the genus *Bradyrhizobium* was supported by comparative analysis of 16S rDNA sequences and the concatenation of *atpD*, *dnaK*, *gyrB*, and *rpoB* gene sequences. MLSA indicated also that *L. nigricans* microsymbionts are members of *Bradyrhizobium japonicum*.

Conclusion *L. nigricans* root nodule symbionts are members of *Bradyrhizobium japonicum* and exhibit high phenotypic and genomic diversity important for their survival in soil.

Keywords AFLP · BOX-PCR · *Bradyrhizobium* · Genisteae · *Lembotropis nigricans* · Multilocus sequence analysis

Introduction

Rhizobia are soil-inhabiting bacteria capable of establishing nitrogen-fixing symbiosis with plant species of the family Fabaceae. Genisteae encompassing more than 600 species distributed among 25 genera is one of the largest tribes within the legume family (Cardoso et al. 2013). There are published reports on the phylogeny and taxonomy of bacterial strains isolated from root nodules of 77 plant species of the tribe Genisteae. Based on these reports, it was concluded that the strains of the genus *Bradyrhizobium* form a predominant group infecting Genisteae legumes (Stępkowski et al. 2018). Still, there are no data available on rhizobia of 14 Genisteae genera. One of such plant genus is *Lembotropis* comprising two species: *Lembotropis nigricans* (L. Griseb.) and *Lembotropis emeriflora* (Rchb.). *L. nigricans* (black broom) is distributed in Central and Southeastern Europe; however, its exact range is hard to estimate since it has been grown as an

Magdalena Wójcik and Michał Kalita contributed equally to this work.

The GenBank accession numbers for sequences generated in this study are as follows: MK183761-MK183771 (16S rRNA), MK202810-MK202820 (*atpD*), MK202821-MK202831 (*dnaK*), MK202832-MK202842 (*gyrB*), MK202843-MK202853 (*rpoB*).

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ornamental shrub for many years and it can currently be found in many areas as a wild plant (Petrowicz 1981). Although it has been previously demonstrated that *L. nigricans* produces cylindrical root nodules (Łotocka et al. 2012), the bacteria residing in these structures have not been analyzed for their phenotypic properties, genomic diversity, and taxonomic affiliation.

Polyphasic taxonomy, which integrates all available genotypic, phenotypic, and phylogenetic information, has been widely used for identification and classification of bacteria since the 1970s (Vandamme and Peeters 2014). Numerical analysis of phenotypic and genotypic features allows differentiation of closely related bacteria and preliminary determination of the genus position of microorganisms. The PCR fingerprinting techniques such as BOX-PCR and AFLP are often used for the assessment of the genomic diversity of rhizobia isolated from root nodules of different legume species (Kalita and Małek 2006; Liu et al. 2011; Gnat et al. 2015; Xu et al. 2016; Wdowiak-Wróbel et al. 2017; Chidebe et al. 2018).

Since the 1990s, the phylogenetic studies of root nodule isolates have been based mainly on partial or complete 16S rDNA sequences (Young et al. 1991; Vinuesa et al. 1998). Since then, many studies have demonstrated that the diversity in the 16S rRNA sequences of rhizobia is low and distinguishes species poorly (Menna et al. 2009; Delamuta et al. 2013). Due to the limitations of the 16S rRNA gene analysis, protein-encoding genes with evolution rates faster than that of 16S rDNA, but conserved sufficiently to retain genetic information, have been proposed as alternative phylogenetic markers. *atpD*, *dnaK*, *glnII*, *gyrB*, *recA*, and *rpoB* are examples of housekeeping genes that are frequently used in tracing the evolutionary history of bacteria (Gaunt et al. 2001; Stępkowski et al. 2003; Vinuesa et al. 2005; Rivas et al. 2009; Kalita and Małek 2017; Huang et al. 2018).

In this study, we examined the genus position and evolutionary history of *L. nigricans* root nodule isolates using numerical analysis of phenotypic properties and comparative sequence analysis of 16S rRNA, *atpD*, *dnaK*, *gyrB*, and *rpoB* genes. *L. nigricans* symbionts were also examined for BOX-PCR and AFLP patterns to study their genomic relationship and diversity.

Materials and methods

Bacterial strains

The strains used in this study and their origin are listed in Supplementary Table S1. Root nodules of *L. nigricans* were collected from plants growing at a single location in southeast Poland (51° 08′ 47.2″ N 23° 24′ 43.3″ E). The *L. nigricans* population occupied area of approximately 50 m^2. Ten randomly selected individual plants from five distinct patches were used for root nodule collection. The rhizobia studied were isolated from root nodules with the standard procedure (Kalita and Małek 2004). Yeast extract mannitol medium (YEM) was routinely used for cultivation of the rhizobia at 28 °C. The strains were stored as source cultures on YEM slants at 4 °C.

Phenotypic characterization and numerical analysis of phenotypic properties

L. nigricans nodule isolates were examined for cell morphology, motility, and generation time according to a procedure described earlier by Wdowiak and Małek (2000). Their phenotypic properties, i.e., temperature and pH growth ranges, NaCl tolerance, acid and alkali production in YEM agar supplemented with bromothymol blue as a pH indicator, utilization of different compounds as sole carbon and nitrogen sources, resistance to antibiotics and dyes, reaction in litmus milk, activity of urease, catalase, β-galactosidase, phosphatase, nitrate reductase, cytochrome oxidase, β-D-glucosidase, and peroxidase, synthesis of indole, melanin, and indole-3-acetic acid (IAA), adsorption of Congo red and Calcofluor, and precipitation of calcium glycerophosphate were determined according to Wdowiak and Małek (2000). The utilization of various compounds as a sole carbon source was studied in modified Bergersen's synthetic medium (BS medium) in which mannitol was replaced by the tested compound. The utilization of various compounds as a sole nitrogen source was examined in the same BS medium with the tested substance instead of NH_4Cl. All phenotypic tests were done in triplicate and repeated two times. The results were scored after 5 days for fast-growing strains and 7–10 days for slow-growing strains (Garrity et al. 2005).

For numerical analysis, the phenotypic features of the rhizobia studied were coded in the binary system. Next, the simple matching similarity coefficient (SM) of each strain pair was estimated and a similarity matrix was generated (Sneath and Sokal 1973). Based on the similarity matrix, clustering analysis was performed with the unweighted pair group method with arithmetic mean (UPGMA) using the NTSYSpc software package (Exeter Software).

Isolation of total genomic DNA

For DNA isolation, bacteria were grown in 30 ml of liquid YEM medium for 4–5 days at 28 °C. DNA was extracted and purified according to the method proposed by Pitcher et al. (1989) as described elsewhere (Kalita and Małek 2004).

BOX-PCR, AFLP, and data analysis

The primers and PCR cycling conditions used in the BOX-PCR and AFLP analyses are listed in Supplementary

Table S2. The AFLP procedure was done as described by Tyrka (2002) with some small modifications as described elsewhere (Kalita and Małek 2006). Each reaction was repeated three times on the same DNA matrix. Only informative and reproducible products of the genomic fingerprinting reactions were analyzed. The PCR products were separated in 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The DNA profiles yielded by the BOX-PCR and AFLP methods were analyzed using BIO-GEN program version 11.01 (Vilber-Lourmat). The strains were grouped by the Nei and Li coefficient (Nei and Li 1979) and the dendrogram was constructed using the UPGMA method.

Amplification and sequencing of 16S rRNA, *atpD*, *dnaK*, *gyrB*, and *rpoB* genes

The primers and PCR amplification conditions used in this study are listed in Supplementary Table S2. All PCR amplification reactions were carried out with ReadyMix™ *Taq* PCR Reaction Mix (Sigma) according to the manufacturer's recommendations. DNA-free water was used in negative PCR controls. Genomic DNA of *B. japonicum* USDA6^T was used as a template in positive PCR controls. The amplified products were purified with Clean-Up or Gel-Out purification columns (A&A Biotechnology) and sequenced with the BigDye Terminator Cycle sequencing kit (Thermo Fisher Scientific) using the 3500 Genetic Analyzer according to the manufacturer's procedures.

Phylogenetic analysis

The sequences yielded in this study were compared to the nucleotide sequences from GenBank database using the BLAST program (Altschul et al. 1990). Since all the sequences were most similar to the sequences of the genus *Bradyrhizobium* strains, only reference bradyrhizobia were included for further phylogenetic analyses. All phylogenetic analyses were conducted in MEGA 7 (Kumar et al. 2016) as follows. Multiple sequence alignments were constructed and the resulting alignments were corrected manually. To obtain the same number of analyzed positions in the alignment, longer sequences were truncated. As a result, 1228, 429, 204, 563, and 452 nucleotide positions were analyzed for the 16S rRNA, *atpD*, *dnaK*, *gyrB*, and *rpoB* genes, respectively. The maximum likelihood (ML) method was used to reconstruct the phylogeny of the analyzed genes. jModelTest (Darriba et al. 2012) was used to choose the best-fit evolutionary model for each studied gene. To determine the degree of the statistical support for the branches in the phylogeny, 1000 bootstrap replicates of the data were analyzed. Sequence identity values for *atpD-dnaK-gyrB-rpoB* concatenation were calculated using BioEdit software (Hall 2011) based on the multiple alignment constructed in MEGA7.

Results and discussion

Phenotypic properties of *L. nigricans* rhizobia

A total of 48 bacterial strains, including 33 *L. nigricans* symbionts and 15 reference strains, representing different species of the genera: *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, and *Rhizobium* were analyzed for 86 phenotypic properties. Table 1 presents some physiological and metabolic properties of the tested bacteria. In Supplementary Table S3, the results of all 86 characteristics are shown. The growth temperature range for the black broom rhizobia was determined to be between 13 and 3 °C with an optimal temperature of 28–30 °C, typical for most rhizobia. All *L. nigricans* symbionts, likewise the reference *Bradyrhizobium* species, grew at pH 5–8. No strain tolerated pH 4. Forty-five percent of the strains were able to grow at pH 9 and 21% grew even at pH 10. The black broom rhizobia were able to grow on YEM medium with 0.5 and 1% NaCl. Most of them tolerated 2% sodium chloride and 32% of the studied isolates tolerated 3% NaCl, in contrast to the genus *Bradyrhizobium* strains, which generally exhibit high sensitivity to salinity (Garrity et al. 2005). The *L. nigricans* symbionts were slow-growing rhizobia with generation time ~ 6 h in YEM broth at 28 °C. Such a doubling time is characteristic for the strains of the genus *Bradyrhizobium* (van Berkum and Eardly 1998). Tests for assimilation of different compounds as sole carbon and nitrogen sources are commonly used in taxonomic studies of rhizobia. In our experiments, 25 different carbon-containing compounds were tested as sole sources of carbon for bacteria. All the tested isolates utilized 12 of the 25 studied carbon sources, i.e., L-alanine, L-arginine, dextrin, mannitol, glycerol, sucrose, Tween-20, D-fructose, D-galactose, insulin, D-xylose, and sodium tartrate. The other carbon compounds served as growth substrates for only some bacteria studied (Table 1). In YEM medium with mannitol as a sole carbon source, black broom symbionts synthesized acid products similar to *Rhizobium*, *Ensifer*, and *Mesorhizobium* species strains (Garrity et al. 2005; Gnat et al. 2014). Most of the tested strains did not utilize disaccharides such as maltose and trehalose similar to slow-growing bradyrhizobia, which generally do not have disaccharide uptake systems (Glenn and Dilworth 1981; Elkan 1992). However, most of them exhibited good growth on lactose and sucrose.

Of the 21 nitrogen sources tested, only DL-ornithine was not utilized by all the *L. nigricans* symbionts, whereas 81 and 79% of the bacteria did not utilize L-lysine and sodium hippurate, respectively. The relevant phenotypic traits that differentiated the *L. nigricans* nodulators and the reference *Bradyrhizobium* species are listed in Table 1. An important taxonomic criterion used in differentiation of fast-growing

Table 1 Some phenotypic characteristics of *Lembotropis nigricans* root nodule isolates and reference *Bradyrhizobium* strains

Characteristics	*Lembotropis nigricans* isolates ($n^a = 33$)	*Bradyrhizobium elkanii* USDA 76^T ($n = 1$)	*Bradyrhizobium liaoningense* USDA 3622^T ($n = 1$)	*Bradyrhizobium yuanmingense* CCBAU 10071^T ($n = 1$)	*Bradyrhizobium diazoefficiens* USDA 110^T ($n = 1$)	*Bradyrhizobium japonicum* USDA 6^T ($n = 1$)
Carbon sources used						
D-Arabinose	+[b] (24)[c]	+	+	+	+	+
D-Cellobiose	+ (21)	+	+	+	+	+
D-Glucose	+ (27)	+	+	+	+	+
D-Raffinose	+ (9)	+	+	+	+	+
D-Trehalose	+ (4)	–	+	+	–	–
D-Xylose	+ (33)	+	–	–	+	+
Dextrin	+ (33)	–	–	+	+	+
Inulin	+ (33)	+	–	–	+	+
Lactose	+ (27)	–	–	–	+	+
L-Alanine	+ (33)	+	+	–	+	+
L-Arginine	+ (33)	+	–	–	+	+
L-Asparagine	+ (24)	+	–	–	+	+
L-Glutamine	+ (29)	+	+	+	+	+
L-Lysine	+ (14)	–	–	–	–	–
L-Rhamnose	+ (14)	+	+	+	+	+
L-Tyrosine	+ (29)	+	–	–	–	–
Maltose	+ (9)	–	+	–	+	+
Salicin	+ (21)	+	–	+	+	+
Sodium citrate	+ (24)	+	–	–	+	+
Sodium hippurate	+ (4)	–	–	–	–	–
Starch	+ (24)	–	–	–	–	–
Nitrogen sources used						
DL-Isoleucine	+ (30)	+	–	+	–	+
L-Glutamic acid	+ (33)	+	+	–	+	+
L-Lysine	+ (6)	+	–	+	–	+
L-Phenylalanine	+ (29)	+	–	–	+	+
L-Serine	+ (30)	+	–	–	+	+
Sodium hippurate	+ (7)	+	–	–	–	–
Sodium nitrate	+ (31)	+	+	+	+	+
Resistant to						
pH 9.0	+ (15)	–	–	–	–	–
pH 10.0	+ (7)	–	–	–	–	–
1.0% NaCl	+ (32)	+	–	–	–	–
3.0% NaCl	+ (11)	–	–	–	–	–
Auramine 0.05 μg ml^{-1}	+ (23)	+	–	+	+	+
Crystal violet 0.1 μg ml^{-1}	+ (2)	–	–	–	+	+
Methyl green 0.13 μg ml^{-1}	+ (5)	–	–	–	–	–
Neutral red 0.2 μg ml^{-1}	+ (24)	–	+	+	+	+
Ampicillin 100 μg ml^{-1}	+ (22)	+	–	–	+	+
Ampicillin 200 μg ml^{-1}	+ (13)	+	–	–	+	+
Rifampicin 20 μg ml^{-1}	+ (28)	+	+	+	+	+

Table 1 (continued)

Characteristics	*Lembotropis nigricans* isolates ($n^a = 33$)	*Bradyrhizobium elkanii* USDA 76^T ($n = 1$)	*Bradyrhizobium liaoningense* USDA 3622^T ($n = 1$)	*Bradyrhizobium yuanmingense* CCBAU 10071^T ($n = 1$)	*Bradyrhizobium diazoefficiens* USDA 110^T ($n = 1$)	*Bradyrhizobium japonicum* USDA 6^T ($n = 1$)
Rifampicin 200 μg ml^{-1}	+ (12)	–	–	–	+	+
Streptomycin 10 μg ml^{-1}	+ (31)	+	+	+	+	+
Streptomycin 200 μg ml^{-1}	+ (13)	+	–	–	–	–
Tetracycline 10 μg ml^{-1}	+ (30)	+	+	+	+	+
Tetracycline 40 μg ml^{-1}	+ (3)	+	–	–	–	–

[a] *n*, the number of studied strains

[b] +, −, strains were positive, negative, respectively

[c] Value in parentheses is the number of strains with positive reaction

from slow-growing rhizobia is their tolerance to antibiotics (Elkan 1992). The black broom nodule isolates showed rather high tolerance to the tested antibiotics, similar to the *Bradyrhizobium* species (Kalita and Małek 2004). Most of them were resistant to ampicillin (100 μg ml^{-1}), rifampicin (20 μg ml^{-1}), streptomycin (10 μg ml^{-1}), and tetracycline (10 μg ml^{-1}). All of them were able to grow in the medium supplemented with acridine orange (0.013%), crystal violet (0.013%), methyl red (0.05%), methyl green (0.065%), neutral red (0.1%), nigrosine (0.5%), and safranin (0.2%). Rhizobia specific to *L. nigricans* were highly homogeneous in enzyme activities (except catalase and phosphatase) and all of them hydrolyzed urea, reduced nitrate, produced active cytochrome oxidase, and exhibited evident alkaline reaction in the litmus milk test.

The phenotypic properties of the *L. nigricans* rhizobia and the reference strains representing the genera *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, and *Rhizobium* were subjected to numerical analysis with the use of the NTSYSpc software. The resulting dendrogram is shown in Fig. 1. The complete binary matrix table used for the numerical analysis is available as Supplementary Table 3. On the basis of the cluster analysis, all strains included into analysis formed two phena at the similarity coefficient level of 0.71. Phenon I included bacteria of the genera *Rhizobium*, *Ensifer*, and *Mesorhizobium*. Phenon II contained slow-growing strains of the genus *Bradyrhizobium* and *L. nigricans* symbionts, which formed a separate subgroup at a similarity coefficient of 0.82. The results presented in the dendrogram indicate that the slow-growing black broom rhizobia belong to the genus *Bradyrhizobium*. The high level of phenotypic diversity of the *L. nigricans* symbionts determined by the numerical analysis may facilitate their survival in changing environmental conditions in which these bacteria naturally live.

BOX-PCR and AFLP analysis

In recent years, many methods have been developed for molecular typing and assessing genomic heterogeneity of bacteria mainly within species or within closely related species. One of the best known genome fingerprinting techniques based on polymerase chain reaction (PCR) is BOX-PCR of highly conserved repetitive DNA sequences occurring naturally in bacterial genomes (Lupski and Weinstock 1992; Versalovic et al. 1994) as well as AFLP based on nucleotide changes within restriction sites and adjacent bases, which serve for primer annealing in the PCR reaction (Blears et al. 1998; Savelkoul et al. 1999). Both techniques generating DNA profiles specific to a given genome were used for analysis of the genomic diversity and genomic relationship of the 33 *L. nigricans* root nodule isolates. In the BOXA1R-PCR method, a 22-base oligonucleotide primer containing 68% of GC was applied. The use of the GC-rich primer in the BOX-PCR method was associated with the high GC content of rhizobial genomes (Garrity et al. 2005) and helped to maximize the number of amplicons. In the PCR reaction with all black broom rhizobia, the BOXA1R primer produced DNA bands ranging in the size from 275 to 2134 nucleotides, with an average number of nine bands per strain. The DNA BOX patterns were used for cluster analysis and presentation of the genomic relationship in the form of a dendrogram (Fig. 2). In the generated dendrogram, the 33 *L. nigricans* symbionts were divided into three main clusters. In the first cluster, two strains (LN1 and LN14) were placed distinctly from the other bacteria. The second main cluster comprised 17 isolates with a DNA pattern similarity level from 71 to 100%, whereas the third one encompassed 14 strains with a DNA pattern similarity level in the range from 82 to 100%. The BOX-PCR method allowed identification of 27 genomotypes among the 33 black broom microsymbionts. Eleven strains belong to 5 clusters

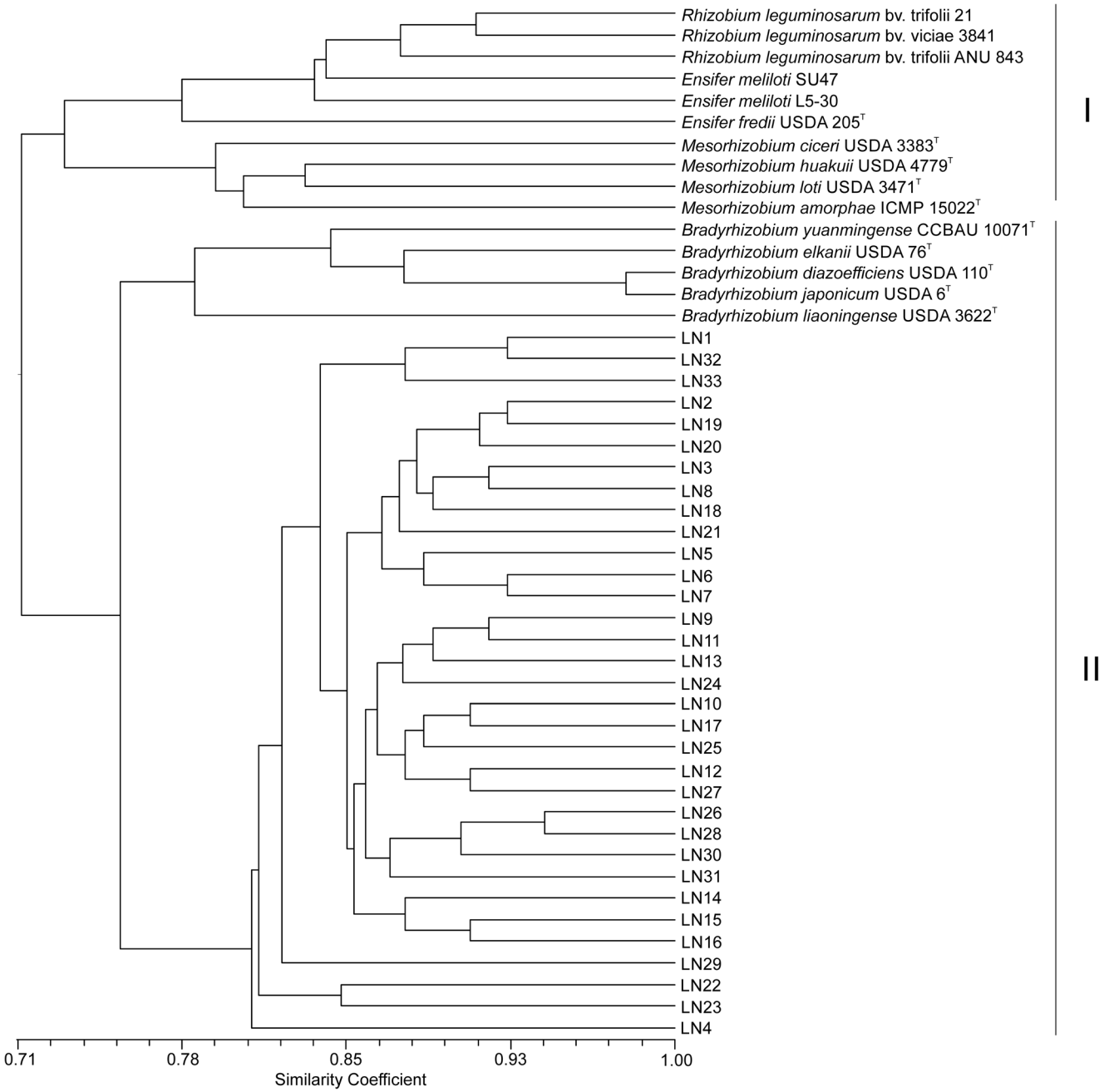

Fig. 1 UPGMA dendrogram showing phenotypic relationships among 33 *Lembotropis nigricans* root nodule isolates and reference strains

comprising at least two root nodule isolates sharing identical DNA profiles (Fig. 2).

To investigate the genomic diversity of the *L. nigricans* symbionts in greater detail, the AFLP technique was also used. Presently, bacteriologists frequently use this method in the assessment of genomic diversity both within species and across different bacterial populations (Aserse et al. 2012; Li et al. 2012; Xu et al. 2016). For fingerprinting the black broom rhizobial genomes and assessment of their genomic relationships with the AFLP method, *Pst*I endonuclease recognizing a GC-rich sequence 5′-CTGGAG-3′ and the primer pair PstI-GC with GC as arbitrary bases at the 3′ end were used. The AFLP profiles of the bacteria studied contained from 1 to 19 DNA bands per strain and their size was from 184 to 1486 bp. On the basis of DNA banding profiles, it was possible to differentiate all black broom rhizobia except two strains, which exhibited identical genomic patterns in the AFLP method with the PsI-GC primer. The genomic relationships of *L. nigricans* symbionts based on AFLP fingerprinting data are presented in the dendrogram generated by UPGMA cluster analysis (Fig. 2). In this tree, the rhizobia studied were split into two major groups at a DNA similarity coefficient level of 0.55. One cluster encompassed ten rhizobia, including two strains (LN22 and LN23) sharing identical DNA patterns.

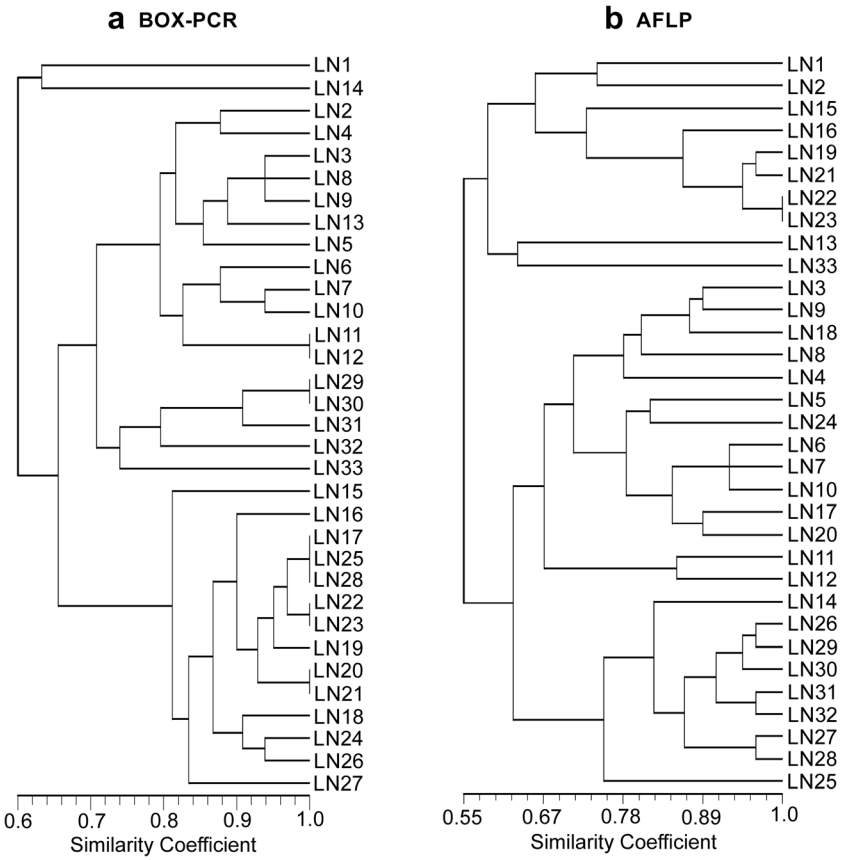

Fig. 2 Dendrograms based on (**a**) BOX-PCR and (**b**) AFLP data of 33 *Lembotropis nigricans* root nodule isolates. Nei and Li coefficient was used for similarity measure. UPGMA was used for clustering

The other group comprised 23 strains separated into two genomically different subgroups at a similarity coefficient level of 0.62. Genome heterogeneity of rhizobia is shaped by environmental factors and limited by the symbiotic interaction with the host plant. Our studies showed that the AFLP technique was superior to the BOX-PCR method in differentiating the *L. nigricans* symbionts and facilitated identification of 32 genomotypes among the 33 bacterial strains studied. We also showed that both these genome profiling techniques used offer a convenient way to choose the representative strains from each genomic group for further taxonomic studies, such as 16S rDNA and multilocus sequence analysis (MLSA).

16S rDNA sequence analysis

Comparative analysis of 16S rDNA sequences is widely used to study the taxonomic position of bacteria at the genus level and to depict bacterial phylogeny. It was demonstrated that a 16S rRNA gene sequence similarity lower than 98.7% suggests that bacterial strains belong to distinct species (Yarza et al. 2014). Analysis of 16S rRNA gene sequences has some difficulties in the case of *Bradyrhizobium* bacteria since many newly described bradyrhizobial species show 99.4% or higher 16S rDNA sequence identity to the previously defined species of the genus *Bradyrhizobium* (Chahboune et al. 2012; Guerrouj et al. 2013; Grönemeyer et al. 2017; Costa et al. 2018).

In order to clarify the genus position of the *L. nigricans* symbionts and investigate their evolutionary relationship with other bacteria of the genus *Bradyrhizobium*, the nearly full-length 16S rRNA encoding genes of 11 symbionts representing different phenotypic and genomic groups of black broom nodule isolates were amplified and sequenced. The 16S rDNA sequences of the studied rhizobia were aligned and compared with those of other nodule bacteria available in the GenBank database. The evolutionary distances between *L. nigricans* symbionts and reference bacteria representing the genus *Bradyrhizobium* were calculated from a 1228 bp-long alignment and molecular phylogeny was reconstructed using the maximum likelihood method (ML). The level of sequence similarity between the 16S rDNA of the black broom rhizobia and those of *Bradyrhizobium* ranged from 94.8 to 100%. The *L. nigricans* rhizobia were most similar (≥ 99.5%) in their 16S rDNA sequences to *B. japonicum* USDA 6^T, *B. canariense* BTA-1^T, *B. liaoningense* USDA 3622^T, *B. daqingense* CCBAU 15774^T, and *B. americanum* CMVU44^T. The 16S rDNA sequences of the black broom nodule isolates shared 99.6–100% sequence identity. The 16S rDNA nucleotide sequences of strains LN1, LN2, LN10, LN11, LN20, LN30, and LN32 were identical to each other and to *B. japonicum* BGA-1. Interestingly, the 16S

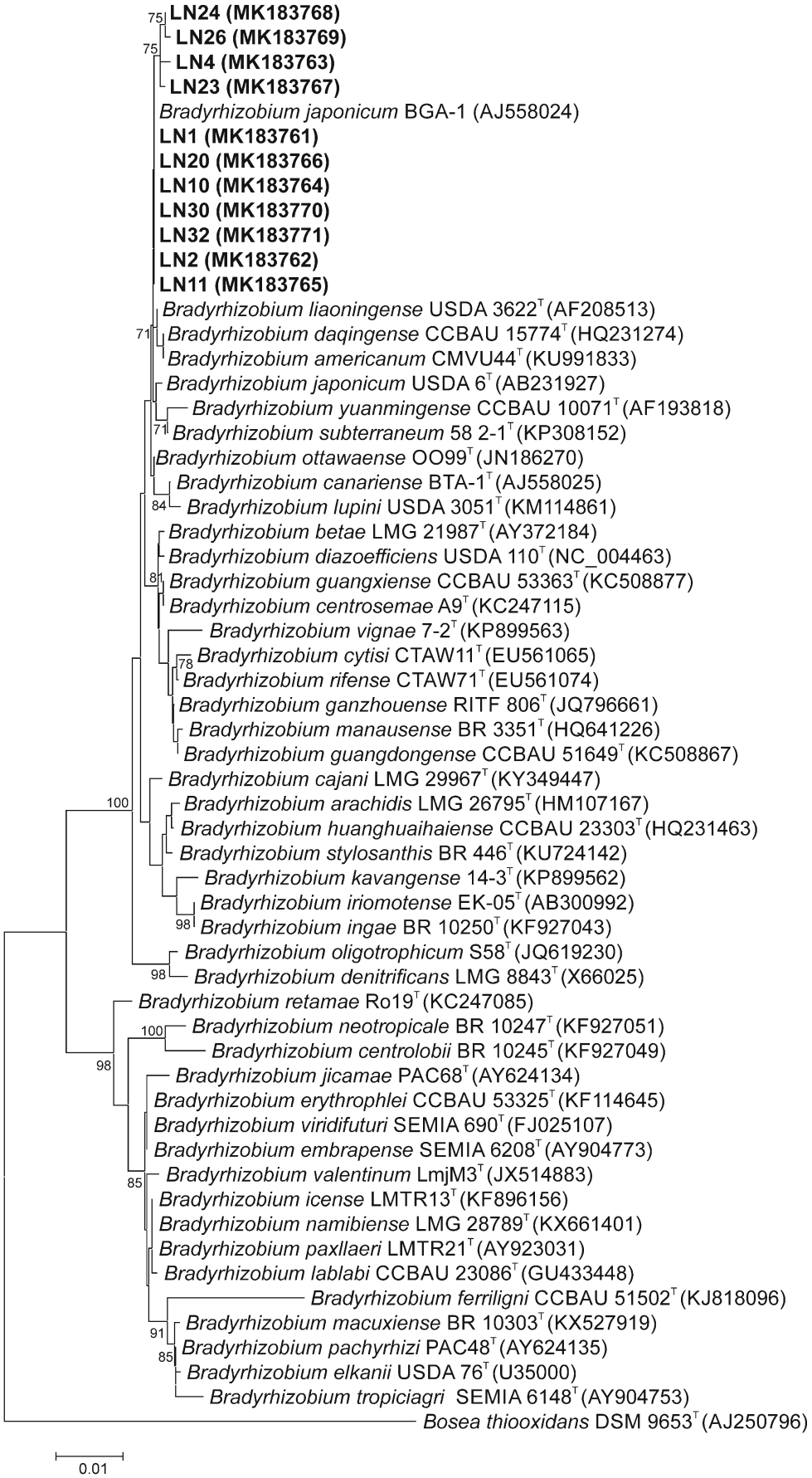

Fig. 3 Maximum likelihood phylogenetic tree of 16S rDNA sequences of *Lembotropis nigricans* root nodule isolates (shown in bold) and reference bradyrhizobia. Bootstrap values ≥ 50% are given at the branching points. The scale bar indicates the number of substitutions per site. GenBank accession numbers are given in parentheses

rDNA sequence of *B. japonicum* BGA-1 was different from that of *B. japonicum* USDA 6^T at two nucleotide positions (99.8% similarity) at which it was identical to *B. liaoningense* USDA 3622^T (99.9% sequence similarity). This observation clearly indicates a very low level of 16S rRNA gene sequence heterogeneity among *Bradyrhizobium* species. It also suggests that 16S rRNA as a molecular marker has serious limitations for species delineation. The results of comparative 16S rDNA sequence analysis confirmed those of the numerical analysis of phenotypic features (Fig. 1) and showed that the black broom rhizobia are members of the genus *Bradyrhizobium* species. The phylogenetic relatedness of the *L. nigricans* rhizobia with other nodule bacteria is presented in the form of a phylogram in Fig. 3. On the 16S rDNA tree, the *L. nigricans* symbionts and reference *Bradyrhizobium* strains were divided into two distinct clusters with bootstrap values of 100% and 98%. The cluster with 100% confidence encompasses all black broom rhizobia and 28 reference bradyrhizobia. The *B. elkanii*, *B. erythrophlei*, *B. valentinum*, and *B. lablabi* species were assigned to the other group. The same splitting of the *Bradyrhizobium* species was previously described for phylogenetic trees reconstructed using 16S rDNA sequences (Menna et al. 2009; Delamuta et al. 2012; Kalita and Małek 2017). The pattern of branching on the phylogram shown in Fig. 3 suggests a close relationship of the studied black broom rhizobia with *B. japonicum* BGA-1. However, due to the high level of 16S rRNA sequence conservation between the *Bradyrhizobium* species described above, where two strains of different species can have more similar 16S rDNA nucleotide sequences than two strains of the same species, additional analyses were used to determine the species position of the *L. nigricans* root isolates.

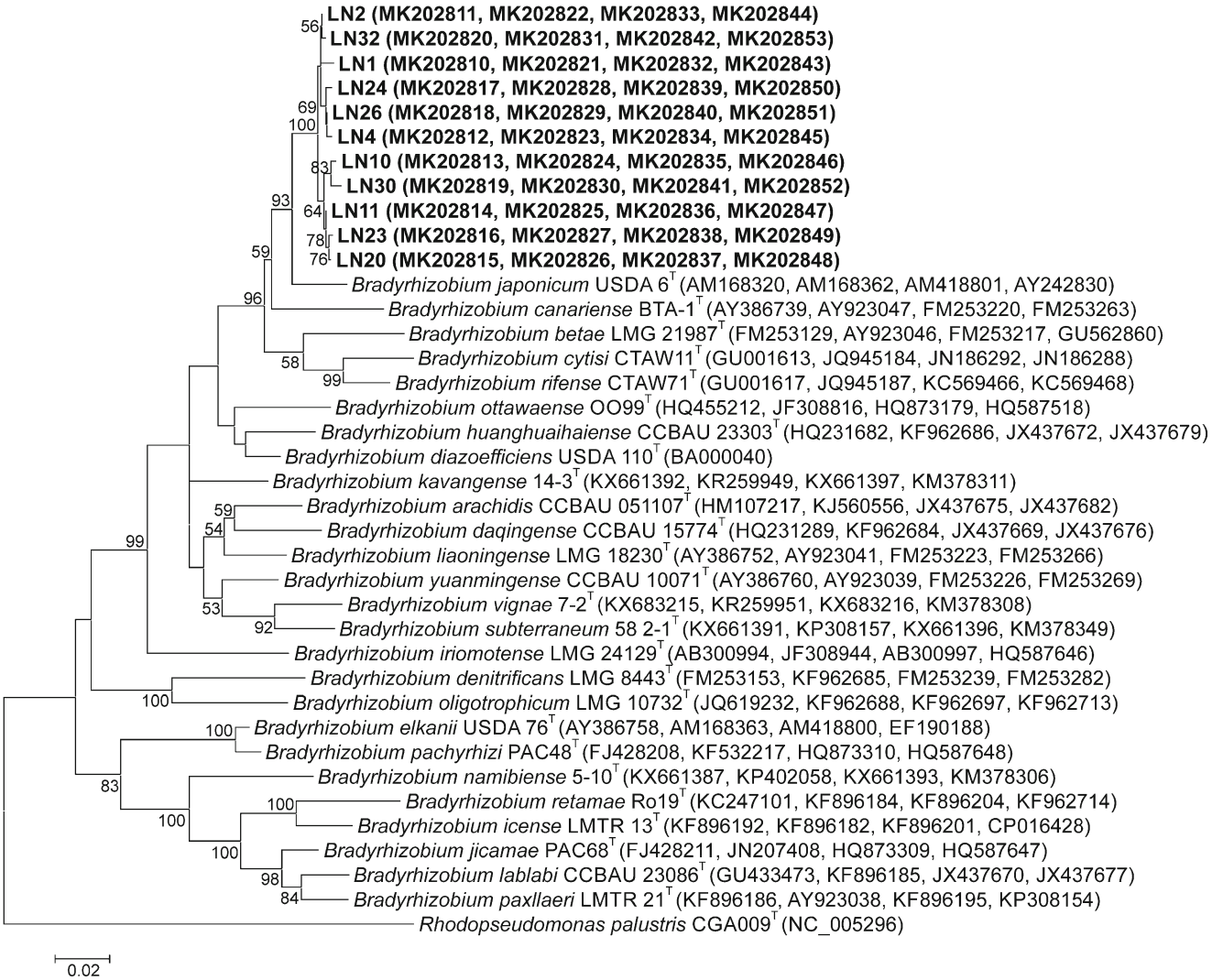

Fig. 4 Maximum likelihood phylogenetic tree of concatenated *atpD*, *dnaK*, *gyrB*, and *rpoB* gene sequences of *Lembotropis nigricans* root nodule isolates (shown in bold) and reference bradyrhizobia. Bootstrap values ≥ 50% are given at branching points. The scale bar indicates the number of substitution per site. GenBank accession numbers are given in parentheses

Analysis of concatenated *atpD-dnaK-gyrB-rpoB* gene sequences

Phylogenetic analysis was carried out using concatenated nucleotide sequences of four housekeeping genes: *atpD*, *dnaK*, *gyrB*, and *rpoB*. Housekeeping genes have been widely used in many studies of *Bradyrhizobium* bacteria to delineate closely related species (Vinuesa et al. 2005; Rivas et al. 2009; Chahboune et al. 2012; Delamuta et al. 2013; Kalita and Małek 2017). Although the protein-encoding genes used in the phylogenetic analysis of *Bradyrhizobium* bacteria display a considerably higher level of sequence diversity compared to 16S rDNA, no threshold value that could be used for species demarcation has ever been proposed as it was done in case of ANI (average nucleotide identity), where it is widely accepted that ≥ 95% ANI represents an accurate threshold for delineating almost all currently named prokaryotic species (Chun et al. 2018; Jain et al. 2018).

The black broom rhizobia shared from 98.9 to 99.8% similarity of concatenated sequences of the four studied genes. The *L. nigricans* strains were most similar to *B. japonicum* USDA 6^T (96.7 to 97.2%). The identity of the analyzed sequences of *L. nigricans* bradyrhizobia to other *Bradyrhizobium* species ranged from 87.7% in the case of *B. retamae* Ro19^T to 95.3% for *B. canariense* BTA-1^T. It should be noticed that the highest similarity value of *L. nigricans* microsymbionts to *B. japonicum* USDA 6^T (97.2%) is lower than the sequence similarity between *B. elkanii* USDA 76^T and *B. pachyrhizi* PAC48^T (98.4%). Moreover, the lowest sequence similarity estimated between *B. japonicum* USDA 6^T and black broom bradyrhizobia (96.7%) overlaps the *atpD-dnaK-gyrB-rpoB* sequence similarity of *B. paxllaeri* LMTR21^T and *B. lablabi* CCBAU23086^T (96.7%). These data indicate that there is no clear gap for interspecific sequence similarity values since, as demonstrated above, two species can exhibit higher identity than strains belonging to a single species. Similar findings were also reported in other studies of the genus *Bradyrhizobium* using different sets of housekeeping genes as molecular markers (Menna et al. 2009; Rivas et al. 2009; Zhang et al. 2012). Regardless of this observation, which could be considered as a drawback of the multilocus sequence analysis (MLSA) in prokaryotic taxonomy, the bacterial strains on the phylogenetic tree reconstructed using concatenated sequences of several genes form clusters supporting their species affiliation. As can be seen on the phylogram in Fig. 4, all the black broom bradyrhizobia form a common group with *B. japonicum* USDA 6^T. This grouping better reflects the evolutionary relationship of the studied isolates with this reference species than analysis based solely on the 16S rDNA sequences. Since there is no information available in the public databases on the *gyrB* gene sequence of *B. japonicum* BGA-1, this strain was not used in our multilocus sequence analysis based on four markers. Nevertheless, the phylogram constructed using concatenation of three gene sequences (*atpD-dnaK-rpoB*) strongly supports the grouping of the *L. nigricans* isolates and *B. japonicum* BGA-1 on the 16S rRNA phylogenetic tree (Supplementary Figure S4). It also supports our conclusion that *L. nigricans* microsymbionts belong to *B. japonicum*.

All the results presented in this study clearly indicate that *L. nigricans*, a plant of tribe Genisteae which has not been previously studied for bacteria inhabiting its root nodules, is infected by strains belonging to *B. japonicum* species. The present study supports our previous results and data reported by other authors that root nodules of the tribe Genisteae plants are mainly inhabited by bacteria of the genus *Bradyrhizobium* (Kalita and Małek 2010; Stępkowski et al. 2011; Kalita and Małek 2017; Stępkowski et al. 2018).

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