Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. Novi Sad, № 132, 49—56, 2017

UDC 633.34:579.84
https://doi.org/10.2298/ZMSPN1732049M

*Jelena B. MARINKOVIĆ*[*], *Dragana Đ. BJELIĆ*, *Branislava B. TINTOR*, *Maja V. IGNJATOV*, *Zorica T. NIKOLIĆ*, *Vojin H. ĐUKIĆ*, *Svetlana N. BALEŠEVIĆ-TUBIĆ*

Institute of Field and Vegetable Crops,
Maksima Gorkog 30, Novi Sad 21000, Serbia

# MOLECULAR IDENTIFICATION OF *Bradyrhizobium japonicum* STRAINS ISOLATED FROM ROOT NODULES OF SOYBEAN (*Glycine max* L.)

ABSTRACT: The aim of this study was to isolate and identify *Bradyrhizobium japonicum* strains on the basis of molecular characteristics. From root nodules of different soybean cultivars were obtained 56 isolates, characterized according to morphological, cultural, and biochemical properties. Among these isolates, 33 isolates showing resemblance with *Bradyrhizobium* sp. were further subjected to molecular identification. Following DNA extraction, a partial 16S rDNA gene sequence from the isolates was amplified by PCR using universal primers fD1 (27F) and rP3 (1492R). Purification and sequencing of the amplified fragments were done in the biotechnology company Macrogen, Seoul, South Korea. Sequences were analyzed using the program FinchTV and BLAST (Basic Local Alignment Search Tool) and compared to sequences in GenBank and the *Bradyrhizobium* ID-database for identification. Comparison of the sequences with the *Bradyrhizobium* ID-database showed that all tested isolates were identified as *Bradyrhizobium japonicum*. Each isolate was deposited in the NCBI GenBank database under a unique accession number. Identification of *Bradyrhizobium* species from root nodules of soybean is of great importance because the symbiosis between rhizobia and legumes are a cheaper and usually more effective agronomic practice for ensuring an adequate supply of nitrogen for legumes, while preserving and improving fertility and productivity of soils.

KEYWORDS: *Bradyrhizobium japonicum*, biological nitrogen fixation, identification, soybean

## INTRODUCTION

Great agricultural, ecological and economic importance of legumes, besides quality and chemical composition of the grain, is reflected in the ability of these plants to fix atmospheric nitrogen in the community with the root nodulating bacteria (Sengupta and Reddy, 2011). Atmospheric nitrogen is converted into plant-available forms through symbiotic nitrogen fixation of legumes and

[*] Corresponding author. E-mail: jelena.marinkovic@ifvcns.ns.ac.rs

bacteria from the family *Rhizobiaceae* (Dixon and Kahn, 2004). Annual return of nitrogen to the soil ranges from 20 to 400 kg per hectare depending on the plant species, bacterial strains and numerous biotic and abiotic factors (Zahran, 1999).

As an important source of proteins and oils in human and animal nutrition, soybean (*Glycine max* L. Merr.) is one of the most cultivated legumes in the world (Nouri *et al.*, 2011). With area exceeding 100,000 ha, soybean is an important factor in the crop production in Serbia (Hrustić and Miladinović, 2008). Nitrogen--fixing bacteria provide "free" nitrogen for soybean plants, increases the yield by 20–50%, and improve the quality of grain without disturbing the natural soil microflora (Milošević and Jarak, 2005).

The most common microsymbionts of soybean are *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* (Zhang *et al*., 2011). The number of *Bradyrhizobium* sp. in our agricultural soils is very small, therefore it is necessary to inoculate legume seeds with nitrogen-fixing bacteria using microbiological inocula (Marinković *et al.,* 2010). Application of microbiological fertilizers containing selected and effective strains of *Bradyrhizobium japonicum* was introduced as a regular measure in the cultivation of soybean (Milošević and Marinković, 2009).

Bacteria that form nodules on the roots of legumes have long been placed in a common genus *Rhizobium*. Nitrogen-fixing bacteria were divided into fast--growing and slow-growing on the basis of culture growth, until Jordan (1982) proposed the separation of slow growing species in a separate genus *Bradyrhizobium*. However, the development and application of molecular techniques in microbiology enabled a simple, fast and reliable genotypic characterization of rhizobia and pointed to their great genetic diversity and divergence. The search for effective strains capable of eliciting and invading root or stem nodules on leguminous plants require isolation and identification of a large number of desirable *Bradyrhizobium* species. Effective strains of *Bradyrhizobium japonicum*, besides the capacity for nitrogen fixation, must also have the competitive ability in relation to the natural population which is most often inefficient in fixing nitrogen (Marinković, 2012).

Therefore, the aim of this study was to perform identification of *Bradyrhizobium* sp. isolated from root nodules of different soybean cultivars on the basis of molecular characteristics.

## MATERIALS AND METHODS

### *Root Nodules Collection*

Nodules were randomly collected from field grown soybean during the four-year period (2010–2013). Four soybean cultivars of medium late and late maturity were selected for the root nodules collection: Balkan (maturity group I), Novosađanka (maturity group I), Venera (maturity group II), and Rubin (maturity group II). Cultivars were obtained from different locations of the Province of Vojvodina, from agricultural fields where soybeans were not previously grown (last five years). All nodules from four plants per each cultivar were separately collected at the full bloom stage of soybean, placed in sterilized polythene bags, transported to the laboratory.

*Isolation of Bradyrhizobium sp.*

Root nodules were surface sterilized and crushed to obtain the bacteria on yeast extract mannitol agar media (YEMA) (Somasegaran and Hoben 1994). Followed by several successive isolations and recultivations of individual pure colonies on the same medium, the isolates were further characterized according to morphological, cultural and biochemical properties (Vincent 1970). Isolates were cultured in yeast extract mannitol broth (YEMB) for 5 days at optimal temperature of 28±2 °C and stocked at 4 °C.

*DNA isolation and PCR analysis*

Isolates showing resemblance with *Bradyrhizobium* sp. were grown on YEMA plates for 72 hrs. DNA was isolated from single bacterial colonies by using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany), following the manufacturer's instructions. For the amplification of 16S rDNA gene fragments, primers fD1 (27F) (AGAGTTTGATCMTGGCTCAG) and rP3 (1492R) (TACGGYTACCTTGTTACGACTT) were used (Weisburg *et al*., 1991). The polymerase chain reaction (PCR) was done in 25-µl aliquots using S-thermal cycler (Eppendorf, Germany) (Table 1).

*Table 1.* PCR protocol

| **Components** | **Final concentration** | **25 µl reaction** |
|---|---|---|
| 2x MMix (Eppendorf) | 1x | 12.5 µl |
| 10 µM Forward Primer | 0.2 µM | 0.5 µl |
| 10 µM Reverse Primer | 0.2 µM | 0.5 µl |
| Template DNA | ~1,000 ng | 1 µl |
| Nuclease-free water | | 10.5 µl |

The PCR reactions were performed with an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, at 55 °C primer annealing for 1 min and at 72 °C extension for 2 min, followed by a final extension step at 72 °C for 3 min (Laguerre *et al*., 1994). Amplicons were electrophoresed in 1.5% agarose gel (Invitrogen) with ethidium bromide. Purification and sequencing of the PCR-amplified DNA fragments were done in the biotechnology company MACROGEN, Seoul, South Korea (http://dna.macrogen.com). FinchTV Version 1.4.0. was used for sequence analysis, and nucleotide sequences were filed in the GenBank Database at the National Center for Biotechnology Information (NCBI).

## RESULTS AND DISCUSSION

When searching for efficient microsymbiotic nitrogen-fixing bacteria, among 56 isolates obtained from different soybean cultivars grown in the Province of Vojvodina, 33 isolates belonged to the genus *Bradyrhizobium*. Based on the morphological characteristics of isolates, species of *Bradyrhizobium* are characterized as rod-shaped, aerobic, non-spore forming and motile by one polar or subpolar flagellum. Colonies are circular, opaque, rarely translucent, white and convex, with entire margins. Strains are usually slow growing, not exceeding 1 mm in diameter within 5–7 days incubation on YEMA, while faster growing strains are uncommon.

Isolates showed negative chemical reaction for indole, methyl red, Voges--Proskauer, hydrogen sulphide production, utilization of carbohydrates and gelatin hydrolysis, and positive reaction for citrate utilization, catalase and ammonia production from peptone and urea (Gachande and Khansole, 2011). Strains are characteristically able to invade the root hairs of leguminous plants and incite the production of root nodules, wherein the bacteria occur as intracellular symbionts with host "specificity" (Gage, 2004). The bacteria are present in root nodules as swollen forms which are normally involved in fixing atmospheric nitrogen into combined forms utilizable by the host plant, while some strains fix nitrogen in the free living state under special conditions (Holt *et al*., 1994).

Characterization of rhizobia based on genetic characteristics is more precise and more informative compared to the morphological and physiological classification. Until 1992, only one species was known within the genus *Bradyrhizobium* – *Bradyrhizobium japonicum* (Jordan, 1982), while the application of molecular methods in the past 20 years enabled the separation of several new species (Ramirez-Bahena *et al.,* 2009).

It has been reported that *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium liaoningense*, *Bradyrhizobium yuanmingense* and *Sinorhizobium fredii* could nodulate soybean. Recently, *Bradyrhizobium huanghuaihaiense*, *Bradyrhizobium daqingense*, *Sinorhizobium sojae*, and several unnamed species were also found to be effective microsymbionts of soybeans (Zhang *et al*., 2011).

In this study, identification of *Bradyrhizobium* isolates based on 16S rDNA homology was performed using PCR with the universal primers 27F and 1492R, probably the most widely used primer pair for amplification of a taxonomically diverse eubacterial 16S rDNA gene fragments by PCR (Weisburg *et al*., 1991). Comparison of the sequences with the *Bradyrhizobium* ID-database showed that all isolates were identified as *Bradyrhizobium japonicum*. BLASTn queries of GenBank and the *Bradyrhizobium* ID-database, showed 100% identity to *B. japonicum* to accessions EU010398.1, KF995085.1, KP219176.1, KC736659.1, JN392462.1, KR092322.1, KX242473.1, CP010313.1, AB680665.1, FJ390915.1, AP012206.1, DQ133343.1, respectively. Isolates were deposited in the NCBI GenBank database under a unique accession number (Table 2).

*Table 2.* Isolates of *Bradyrhizobium japonicum* from root nodules of soybean

| Isolate Code | Soybean Cultivar | Region of Origin | Year of Isolation | Acc. No |
|---|---|---|---|---|
| Bj1 | Balkan | Rimski Šančevi | 2010 | KY000628 |
| Bj2 | Balkan | Rimski Šančevi | 2010 | KY000629 |
| Bj3 | Balkan | Bačka Topola | 2010 | KY000630 |
| Bj4 | Balkan | Srbobran | 2010 | KY000631 |
| Bj5 | Balkan | Rimski Šančevi | 2011 | KY000632 |
| Bj6 | Balkan | Srbobran | 2011 | KY000633 |
| Bj7 | Balkan | Bačka Topola | 2011 | KY000634 |
| Bj8 | Balkan | Sombor | 2011 | KY000635 |
| Bj9 | Novosađanka | Sombor | 2010 | KY000636 |
| Bj10 | Novosađanka | Karavukovo | 2010 | KY000637 |
| Bj11 | Novosađanka | Pančevo | 2010 | KY000638 |
| Bj12 | Novosađanka | Rimski Šančevi | 2010 | KY000639 |
| Bj13 | Novosađanka | Pančevo | 2011 | KY000640 |
| Bj14 | Novosađanka | Hajdučica | 2011 | KY000641 |
| Bj15 | Novosađanka | Srbobran | 2011 | KY000642 |
| Bj16 | Novosađanka | Karavukovo | 2011 | KY000643 |
| Bj17 | Novosađanka | Sremska Mitrovica | 2011 | KY000644 |
| Bj18 | Venera | Sremska Mitrovica | 2012 | KY000645 |
| Bj19 | Venera | Bačka Topola | 2012 | KY072854 |
| Bj20 | Venera | Ruma | 2012 | KY072855 |
| Bj21 | Venera | Sombor | 2012 | KY072856 |
| Bj22 | Venera | Vršac | 2013 | KY072857 |
| Bj23 | Venera | Plavna | 2013 | KY072858 |
| Bj24 | Venera | Plavna | 2013 | KY072859 |
| BJ25 | Venera | Rimski Šančevi | 2013 | KY072860 |
| Bj26 | Rubin | Sombor | 2012 | KY072861 |
| Bj27 | Rubin | Zrenjanin | 2012 | KY072862 |
| Bj28 | Rubin | Kikinda | 2012 | KY072863 |
| Bj29 | Rubin | Rimski Šančevi | 2012 | KY072864 |
| Bj30 | Rubin | Subotica | 2013 | KY072865 |
| Bj31 | Rubin | Zrenjanin | 2013 | KY072866 |
| Bj32 | Rubin | Hajdučica | 2013 | KY072867 |
| Bj33 | Rubin | Rimski Šančevi | 2013 | KY072868 |

Partial and complete sequencing of 16S rRNA made a significant step in the phylogeny and classification of rhizobia, and allowed description of several new genera and species (Germano *et al*., 2006). However, the conservative nature of 16S rRNA gene allows the characterization to the species level, while the differences between the strains of the same species cannot be determined.

More molecular procedures enable the identification and classification of bacteria at a high level of taxonomic resolution, such as using rep-PCR genomic fingerprinting to achieve genetic differences at subspecies and strain levels (Melchiorre *at al*., 2011). Unlike the 16S rRNA gene region, intergenic region 16S-23S rRNA (ITS) shows a high degree of variation among different strains. Variability in the sequences and length of ITS region proved to be very informative in taxonomic evaluation and characterization of indigenous *Bradyrhizobium* populations (Tan *et al*., 2001).

## CONCLUSION

The research confirmed the presence of indigenous *Bradyrhizobium japonicum* in root nodules collected from different soybean cultivars. Further identification using rep-PCR genomic fingerprinting will be necessary to establish genetic differences at the strain level. Also, the selection of strains through inoculation assays in greenhouse and field conditions is needed in order to determine their efficiency in soybean production.

## ACKNOWLEDGEMENTS

This research was supported by the Provincial Secretariat for Higher Education and Scientific Research, Vojvodina, Serbia, Project No. 114-451-2739/2016 (2016–2019): „Sustainable production of plant proteins: soybean, microorganisms, response to climate changes“, and by the Ministry of Education, Science and Technological Development of the R. Serbia (Project No. 31022).

## REFERENCES

Dixon R, Kahn D (2004): Genetic regulation of biological nitrogen fixation. *Nat. Rev. Microbiol.* 2: 621–631.

Gachande BD, Khansole GS (2011): Morphological, cultural and biochemical characteristics of *Rhizobium japonicum* syn. and *Bradyrhizobium japonicum* of soybean. *Biosci. Discov. J.* 2: 1–4.

Gage D (2004): Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol. Biol. Rev.* 68: 280–300.

Germano MG, Menna P, Mostasso FL, Hungria M (2006): RFLP analysis of the rRNA operon of a Brazilian collection of bradyrhizobial strains from 33 legume species. *Int. J. Syst. Evol. Microbiol.* 56: 217–229.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994): *Bergey's Manual of determinative bacteriology*, ninth ed. Williams and Wilkins Pub., MD, USA.

Hrustić M, Miladinović J (2008): Značaj, poreklo i širenje soje. In: Miladinović J, Hrustić M, Vidić M (eds.), *Soja*, Institut za ratarstvo i povrtarstvo, Novi Sad, Sojaprotein, Bečej, 13–43.

Jordan DC (1982): Transfer of *Rhizobium japonicum* Buchanan 1980 to Bradyrhizobium gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *Int. J. Syst. Bacteriol.* 32: 136–139.

Laguerre G, Allard MR, Revoy F, Amarger N (1994): Rapid Identification of Rhizobia by Restriction Fragment Length Polymorphism Analysis of PCR-Amplified 16S rRNA Genes. *Appl. Environ. Microbiol.* 60: 56–63.

Marinković J (2012): „Biohemijska i molekularna karakterizacija simbioze soje i različitih sojeva *Bradyrhizobium japonicum* Kirchner (Jordan) u uslovima suše". Doktorska disertacija, Univerzitet u Novom Sadu, Novi Sad.

Marinković J, Mrkovački N, Aćimović R, Đorđević V (2010): Effect of NS-Nitragin application on soybean yield and yield components agriculture. *Field Veg. Crop Res.* 47: 545–548.

Melchiorre M, de Luca MJ, Anta GG, Suarez P, Lopez C, Lascano R, Racca RW (2011): Evaluation of bradyrhizobia strains isolated from field-grown soybean plants in Argentina as improved inoculants. *Biol. Fertil. Soils* 47: 81–89.

Milošević N, Jarak M (2005): Značaj azotofiksacije u snabdevanju biljaka azotom. In: Kastori R (ed.), *Azot: agrohemijski, agrotehnički, fiziološki i ekološki aspekti*, Naučni institut za ratarstvo i povrtarstvo, Novi Sad, 307–352.

Milošević N, Marinković J (2009): Rizobiumi – biođubriva u proizvodnji leguminoza. *Zbornik radova*, Institut za ratarstvo i povrtarstvo, Novi Sad, 46: 45–54.

Nouri MZ, Toorchi M, Komatsu S (2011): Proteomics approach for identifying abiotic stress responsive proteins in soybean. In: Sudaric A (ed.): *Soybean – molecular aspects of breeding.* InTech, 187–214.

Ramirez-Bahena MH, Peix A, Rivas R, Camacho M, Rodriguez-Navarro DN, Mateos PF, Martinez-Molina E, Willems A, Velazquez E (2009): *Bradyrhizobium pachyrhizi* sp. nov. and *Bradyrhizobium jicamae* sp. nov., isolated from effective nodules of *Pachyrhizus erosus*. *Int. J. Syst. Evol. Microbiol.* 59: 1929–1934.

Sengupta D, Reddy AR (2011): Water deficit as a regulatory switch for legume root responses. *Plant Signal. Behav.* 6: 914–917.

Somasegeran P, Hoben HJ (1994): Handbook for Rhizobia: methods in legume-*Rhizobium* technology, Springer-Verlang, New York.

Tan Z, Hurek T, Vinuesa P, Müller P, Ladha JK, Reinhold-Hurek B (2001): Specific detection of *Bradyrhizobium* and *Rhizobium* strains colonizing rice (*Oryza sativa*) roots by 16S-23S ribosomal DNA intergenic spacer-targeted PCR. *Appl. Environ. Microbiol.* 67: 3655–3664.

Vincent JM (1970): *A manual for the practical study of the root nodule bacteria*. Int. Biol. Prog. Handbook, vol. 15. Blackwell Scientific Publications, Oxford, USA.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991): 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173: 697–703.

Zahran HH (1999): Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* 63: 968–989.

Zhang YM, Li Y Jr, Chen WF, Wang ET, Tian CF, Li QQ, Zhang YZ, Sui XH, Chen WX (2011): Biodiversity and biogeography of rhizobia associated with soybean plants grown in the North China Plain. *Appl. Environ. Microbiol.* 77: 6331–6342.

# МОЛЕКУЛАРНА ИДЕНТИФИКАЦИЈА *Bradyrhizobium japonicum* СОЈЕВА ИЗОЛОВАНИХ ИЗ КОРЕНСКИХ КВРЖИЦА СОЈЕ (*Glycine max* L.)

Јелена Б. МАРИНКОВИЋ, Драгана Ђ. БЈЕЛИЋ, Бранислава Б. ТИНТОР, Маја В. ИГЊАТОВ, Зорица Т. НИКОЛИЋ, Војин Х. ЂУКИЋ, Светлана Н. БАЛЕШЕВИЋ ТУБИЋ

Институт за ратарство и повртарство
Максима Горког 30, Нови Сад 21000, Србија

РЕЗИМЕ: Циљ овог рада је изолација и молекуларна идентификација сојева *Bradyrhizobium japonicum*. На основу морфолошке и биохемијске карактеризације, од 56 изолата из коренских квржица различитих сорти соје, 33 изолата за које је утврђена сличност с *Bradyrhizobium* sp. били су предмет даље идентификације. Након екстракције ДНК, парцијална 16S rDNA генска секвенца из изолата је умножена PCR методом употребом универзалних прајмера fD1 (27F) и rP3 (1492P). Пречишћавање и секвенционирање умножених фрагмената урађено је у компанији Macrogen Ltd. (Сеул, Јужна Кореја). Помоћу програма FinchTV и BLAST (Basic Local Alignment Search Tool) анализе, извршено је вишеструко поређење добијених секвенци с GenBank базом података. Поређењем добијених секвенци с *Bradyrhizobium* ID-базом података сви испитивани изолати идентификовани су као *Bradyrhizobium japonicum*. Секвенце су депоноване у светску NCBI базу уз добијање приступног броја (NCBI Acc. number). Идентификација врста *Bradyrhizobium*-а пореклом из коренских квржица соје од великог је значаја јер је симбиоза између ризобиума и легуминоза исплативији и обично ефикаснији начин снабдевања биљака азотом, а важно је и због очувања и унапређења плодности и продуктивности земљишта.

КЉУЧНЕ РЕЧИ: *Bradyrhizobium japonicum*, биолошка фиксација азота, идентификација, соја