AGRICULTURAL BIOLOGY,
2018, V. 53, № 6, pp. 1285-1293
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 579.64:631.461.52

doi: 10.15389/agrobiology.2018.6.1285eng
doi: 10.15389/agrobiology.2018.6.1285rus

ISOLATION AND IDENTIFICATION OF ROOT NODULE BACTERIA FROM GUAR *Cyamopsis tetragonoloba* (L.) Taub.

I.G. KUZNETSOVA[1], A.L. SAZANOVA[1], V.I. SAFRONOVA[1], J.P. POPOVA[1], D.V. SOKOLOVA[2], N.Yu. TIKHOMIROVA[1], Yu.S. OSLEDKIN[1], D.S. KARLOV[1], A.A. BELIMOV[1, 2]

[1]*All-Russian Research Institute for Agricultural Microbiology,* 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail kuznetsova_rina@mail.ru, v.safronova@rambler.ru (✉ corresponding author), anna_sazanova@mail.ru, elestd@yandex.ru, arriam2008@yandex.ru, arriam2008@yandex.ru, makondo07@gmail.com, belimov@rambler.ru;
[2]*Federal Research Center Vavilov All-Russian Institute of Plant Genetic Resources,* 42-44, ul. Bol'shaya Morskaya, St. Petersburg, 190000 Russia, e-mail dianasokol@bk.ru
ORCID:
Kuznetsova I.G. orcid.org/0000-0003-0260-7677
Sazanova A.L. orcid.org/0000-0003-0379-6975
Safronova V.I. orcid.org/0000-0003-4510-1772
Popova J.P. orcid.org/0000-0003-1570-613X
Sokolova D.V. orcid.org/0000-0002-9967-7454
Tikhomirova N.Yu. orcid.org/0000-0002-8510-2123
Osledkin Yu.S. orcid.org/0000-0001-8865-7434
Karlov D.S. orcid.org/0000-0002-9030-8820
Belimov A.A. orcid.org/0000-0002-9936-8678

The authors declare no conflict of interests
Acknowledgements:
The authors are grateful to P.A. Belimova for valuable assistance in pot trials.
Supported financially by Russian Ministry of Science and Education (project RFMEFI60417X0168, Agreement No. 14.604.21.0168). Long-term storage of strains is supported by the Program for the development and inventory of bioresource collections
Received July 24, 2018

Abstract

Cyamopsis tetragonoloba (guar) belongs to the family *Fabaceae* and is one of the promising crops for cultivation in Russia. Beans contain a large number of protein and fatty oil content, green beans can serve as a valuable source of food and feeds (as seed flour and not ground granulated feeds), but the plant is more in demand as a source of guar gum, which is a polysaccharide formed by galactose and mannose (galactomannan) and is contained in the endosperm of the seeds of this plant. Guar gum is widely used in various industries: food, textile, cosmetic, oil and other. Guar comes from India, where approximately 80 % of the world's production of guar gum is obtained. However, due to high demand, the plant is cultivated throughout the world in areas with a suitable climate (the USA, Sudan, Kenya, Pakistan, Australia), including in the south of the Russian Federation. It is known that the productivity of leguminous crops depends not only on climatic conditions, but also on the effectiveness of symbiosis of plants with nodule bacteria (rhizobia), which is determined by the nitrogen-fixing activity and competitiveness of strains, as well as their complementarity to a particular variety. The use of rhizobia for inoculation of plants is especially important when they are introduced to new habitats, so knowledge of its microsymbionts is necessary for successful cultivation of guar in Russia. This paper is the first to report on isolation of the nodule bacteria of the species *Bradyrhizobium elkanii* from root nodules of the guar plants grown in a pot experiment with the use of soil samples from India. We determined the taxonomic position and genetic heterogeneity of the isolated strains. The 16S rRNA gene (*rrs*), ITS-region between the 16S and 23S rDNA and three "housekeeping" genes *atpD*, *dnaK* and *recA* of 10 isolates of nodule bacteria were sequenced. According to the results of the *rrs* sequence analysis, all isolates are assigned to the species *Bradyrhizobium elkanii* (family *Bradyrhizobiaceae*), whose representatives are microsymbionts of a wide range of leguminous plants, including the tribe *Indigofereae*, to which the guar belongs. However, the representatives of the species were not previously described as a microsymbiont of *Cyamopsis tetragonoloba*. Sequencing of the ITS-region and the "housekeeping" genes confirmed the species identity of the isolates and demonstrated their genetic heterogeneity. Thus, the study of nodule bacteria from guar has expanded our knowledge of the phylogeny of its microsymbionts and will allow us in the future to select the most effective strains that improve nitrogen nutrition and plant growth. Knowledge of the rhizobial microsymbionts of guar will help maximize the symbiotic potential of this agronomically valuable culture for its stable and highly productive cultivation.

Keywords: *Cyamopsis tetragonoloba*, guar, root nodule bacteria, 16S rRNA gene, ITS region,

Cyamopsis tetragonoloba (L.) Taub. (guar, pea tree, or Indian acacia) is an annual leguminous crop with a high content of protein from the tribe *Indigofereae* of the family *Fabaceae*. Besides *C. tetragonoloba*, the genus *Cyamopsis* includes 4 more species (*C. dentata, C. psoraloides*, *C. senegalensis* and *C. serrate*) with a smaller industrial value [1, 2]. It is grown as a vegetable crop and can be used as forage for animals. Guar is a nitrogen-fixing plant; it also serves as a good predecessor in crop rotation. Guar natural gum has a certain importance, which is used as a natural stiffener and emulsifying agent in food, medical, textile and pulp-and-paper industries, in the production of cosmetics, explosives, and as a high-viscosity surfactant in the coal and oil-and-gas industries [3]. The demand for it shows a stable growth: according to the data provided for 2014-2016, the annual requirement for natural gum is about 1.5 million tons, and in 2016, the import of guar natural gum to Russia exceeded 15 thousand tons [4]. The native land of *Cyamopsis* and, accordingly, the basic supplier of guar natural gum is India, though the plant is also cultivated in Pakistan, Sudan, Africa, Australia, Ceylon, Afghanistan and the USA [1]. In Russia, guar was delivered in the mid-twenties [5], but it did not find wide applicability because of insufficient knowledge in the technology of its cultivation [6]. Recent years have been noted for the interest in guar industrial cultivation in the North Caucasian Region of Russia, in the Krasnodar Territory, the Rostov Region and in Crimea [3].

It is known that the productivity of leguminous crops depends on the efficacy of their symbiosis with nodule bacteria, which is defined by nitrogen-fixing activity, virulence, competitiveness, and complementarity (specificity) of strains of microsymbionts to a particular variety of the plant. The application of active strains as inoculates provides intensive nitrogen-fixing, promotes photosynthesis intensifying and, as a consequence, leads to the augmentation of plants productivity [7]. The use of rhizobia for inoculation is especially important in the cultivation of leguminous crops when they are introduced to new habitats, where there are no necessary microsymbionts in the soil. For example, the attempt of cultivation of cultural soya (*Glycine max*) in geographical regions of Russia, atypical for the variety, showed that nodules on roots were practically not formed, and the increase in crop yield and the protein content in vegetative mass and grain demanded introducing specific microsymbionts with seeds [8]. According to past years' research, inoculation of guar growing in Sudan with the strains of *Bradyrhizobium* spp. made an appreciable positive impact on the development of plants, greatly increased the number of nodules, dry mass of plants, nitrogen general content, and seed yield [9].

We believe that successful guar introduction in Russia requires (along with studying suitable research of soil-environmental conditions and development of technologies of cultivation) research on microsymbionts of this crop and the subsequent selection of effective strains.

This paper is the first to report on the isolation of the rhizobia species *Bradyrhizobium elkanii* from the guar nodules. Also, we have defined the taxonomic position and genetic heterogeneity of the isolated strains by sequencing 16S ribosomal DNA, ITS-region and "housekeeping" genes *atpD, dnaK* and *recA*.

The work purpose is obtaining and phylogenetic analysis of microsymbionts of plants *Cyamopsis tetragonoloba*.

Techniques. Seeds of guar (obtained from Vavilov All-Russian Institute of Genetic Resources of Plants, St.-Petersburg) were scarified and superficially sterilized in 98 % H_2SO_4 during 10 min, then carefully washed with sterile tap water and couched on filter paper in Petri dishes at 25 °C in a dark room for 3 days. Seedlings were transferred into plastic pots (12 pots, 4 seedlings per each) with

100 g of sterile vermiculite. Aqueous extracts were prepared from 12 soil samples collected in the state Rajasthan (India), and a 5 ml extract was added per pot.

Plants were grown during 45 days in a phyto-room with a 60 % relative humidity at a 4-level light exposure and temperature mode: night (darkness, 18 °C, 8 h), morning (200 μmol · m^{-2} · s^{-1}, 20 °C, 2 h), day (400 μmol · m^{-2} · s^{-1}, 23 °C, 12 h), and evening (200 μmol · m^{-2} · s^{-1}, 20 °C, 2 h), lamps L 36W/77 FLUORA (Osram, Germany). After the end of the experiment, the roots of the plants were taken out from vermiculite and washed thoroughly with tap water. The formed nodules were separated from the roots, superficially sterilized for 1 min in 70 % ethanol and homogenized in sterile tap water.

Strains of rhizobia were traditionally isolated [10] from the nodule homogenates with the use of modified mannitol-yeast agar YMSA [11] with 0.5 % succinic acid. Strains were cultured at 28 °C. All isolates are deposited to the Russian Collection of Agricultural Microorganisms (RCAM, the Station of the Low-Temperature Computerized Storage of Biological Samples, Liconic Instruments, Liechtenstein) [12]. The information on strains is accessible in the RCAM Internet database [13].

Rhizobia strains were phylogenetically assessed based on sequencing 16S rRNA gene (*rrs*) and ITS-region between 16S and 23S rRNA genes. The primer pairs used were fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′)—rD1 (5′-AAGGAGGTGATCCAGCC-3′) for 16S rDNA gene amplification (a fragment of about 1500 bp), and FGPL-132 (5′-CCGGGTTTCCCCATTCGG-3′)—FGPS1490-72 (5′-TGCGGCTGGATCCCCTCCTT-3′) for ITS-region (800 bp). For more correct taxonomic identification and study of the genetic heterogeneity of strains, "housekeeping" genes *atpD*, *recA* and *dnaK* were sequenced. Amplification of "housekeeping" genes was carried with primers atpD352F and atpD871R, recA63FD and recA504RD [14], dnaK1466Fd and dnaK1777Rd [15]. The PCR products were extracted from gel and refined [16] for sequencing (genetic analyzer ABI PRISM 3500xl, Applied Biosystems, USA). Homologous sequences were searched in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) with BLAST software [17]. A phylogenetic tree was constructed with MEGA5 software using the Neighbor-Joining method [18]. Pairs of sequences were compared according to the number of differing nucleotides. To estimate levels of clusters support, the bootstrap-analysis was carried out (1000 replicates). The obtained sequences are deposited in the GenBank database under the numbers MH938226-MH938235 for *rrs* gene, MH938704-MH938713 for ITS-region, MH982271-MH982280 for gene *atpD*, MH982261-MH982270 for gene *dnaK* and MH982251-MH982260 for gene *recA*.

Results. Ten bacterial strains were isolated from the root nodules of guar, a strain per soil sample. In two samples, nodules were not found on the roots of plants. According to 16S rRNA gene sequencing, all strains are assigned to the species *Bradyrhizobium* and form a monophyletic statistically authentic cluster with a 99 % support level (Fig. 1). In addition to isolates, the cluster included typical strains *B. elkanii* USDA 76T, *B. jicamae* PAC68T, *B. lablabi* CCBAU 23086T, *B. pachyrhizi* PAC48T and *B. tropiciagri* SEMIA 6148T. However, the *rrs* homology of new isolates was maximum (100 %) only with two typical strains, the *B. elkanii* USDA 76T and *B. pachyrhizi* PAC48T (Table). As for the strain *B. tropiciagri* SEMIA 6148T, the *rrs* gene similarity was lower and varied from 98.4 to 99.3 %; *rrs* homology for *B. jicamae* PAC68T and *B. lablabi* CCBAU 23086T was below 99 % (see Table).

To more correctly identify taxonomic position of the isolates and study their genetic heterogeneity, the analysis of three "housekeeping" genes sequences was carried out: *atpD* and *recA*, coding β-subunit of membrane ATP synthase

and DNA recombinase, respectively [14], and *dnaK* which codes chaperone preventing protein aggregation and providing their refolding upon thermal damage [15]. Figure 2 provides a dendrogram constructed on the basis of *atpD*, *dnaK* and *recA* nucleotide sequences.

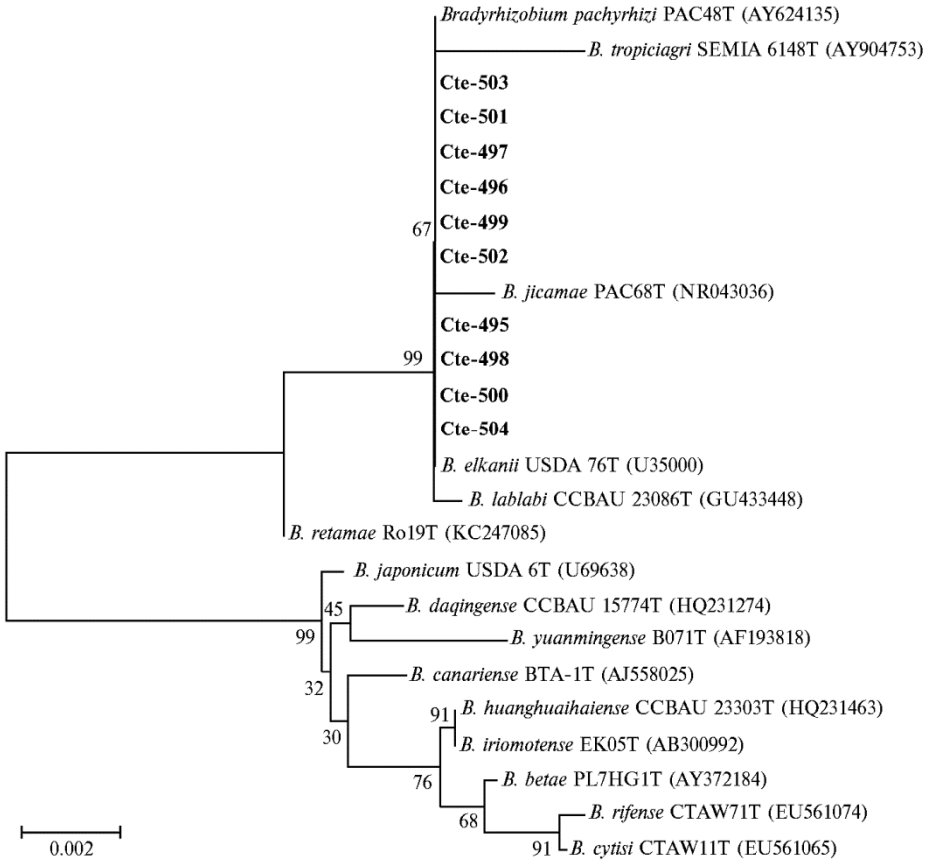

Fig. 1. *rrs*-Phylogram of isolates from guar *Cyamopsis tetragonoloba* nodules and the members of related *Bradyrhizobium* species. Typical strains are marked with the letter T. Guar strains are marked with bold (the Neighbor-Joining method).

Gene sequence homology (%) of isolates from guar *Cyamopsis tetragonoloba* nodules and the closest typical strains of genus *Bradyrhizobium*

Typical strains	Locus	Iso;ate C									
		495	496	497	498	499	500	501	502	503	504
B. elkanii USDA76T	*rrs*	100	100	100	100	100	100	100	100	100	100
	ITS	92.2	93.0	92.3	92.6	92.9	92.1	94.2	91.3	94.3	94.3
	atpD	96.1	96.5	97.4	97.8	97.8	96.8	96.0	96.2	96.3	97.0
	dnaK	98.8	98.8	98.4	97.9	98.4	98.7	98.4	98.7	98.7	99.2
	recA	94.4	95.0	94.5	94.5	94.7	94.5	95.7	94.4	96.1	94.6
B. pachyrhizi PAC48T	*rrs*	100	100	100	100	100	100	100	100	100	100
	ITS	90.6	89.8	90.6	90.7	90.8	90.2	87.9	90.6	87.9	88.3
	atpD	96.1	95.3	95.1	95.7	95.1	94.9	95.7	95.8	95.8	95.1
	dnaK	99.6	99.6	99.2	98.6	99.1	99.0	98.7	99.3	99.0	99.2
	recA	93.8	93.9	93.9	93.9	93.6	93.8	94.8	93.9	94.7	93.6
B. tropiciagri SEMIA6148T	*rrs*	99.3	99.3	98.8	99.0	99.1	99.2	98.4	99.3	99.3	99.2
	ITS	92.1	91.9	91.8	91.5	91.9	91.5	91.4	91.1	90.8	90.8
	atpD	96.9	95.7	95.7	96.2	96.3	95.3	96.6	96.8	96.8	95.3
	dnaK	96.6	96.7	96.3	96.7	96.8	96.7	96.1	97.0	96.4	96.5
	recA	94.7	95.2	95.2	94.8	94.4	94.8	95.5	94.6	95.9	94.4

All isolates were clustered at 100 % support level together with typical strains *B. elkanii* USDA 76T, *B. pachyrhizi* PAC48T and *B. tropiciagri* SEMIA

6148T. Within this group, there are two statistically authentic subclusters formed by isolates Cte-501 and Cte-503, and Cte-495 and Cte-502 (support levels of subclusters are 100 and 98 %, respectively). The maximum homology on the gene *atpD* between new isolates and typical strains was 97.8 % for *B. elkanii*, 96.9 % for *B. tropiciagri* and 96.1 % for *B. pachyrhizi* (see Table). The homology with *dnaK* varied from 98.4 to 99.2 % for *B. elkanii*, from 98.7 to 99.6 % for *B. pachyrhizi* and from 96.1 to 97.0 % for *B. tropiciagri*. According to *recA*, the maximum homology between isolates and typical strains was 96.1 % for *B. elkanii*, 95.9 % for *B. tropiciagri* and 94.8% for *B. pachyrhizi* (see Table 1).

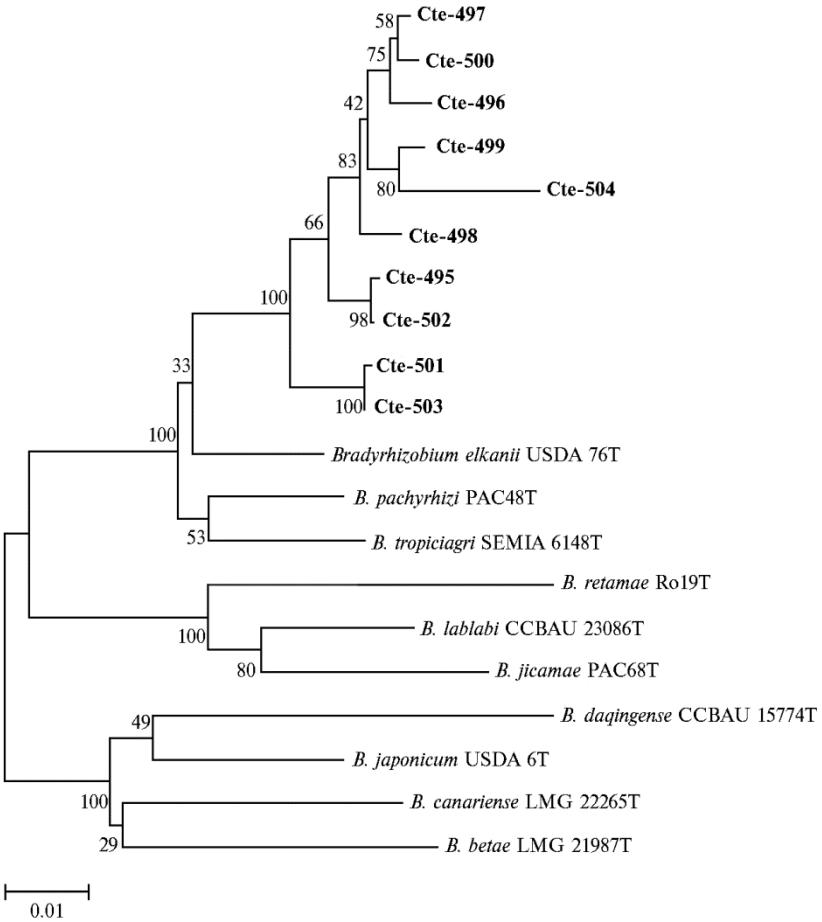

Fig. 2. Phylogram constructed for *atpD, dnaK* and *recA* genes of the isolates from the guar *Cyamopsis tetragonoloba* nodules and the members of related *Bradyrhizobium* species. Typical strains are marked with the letter T. Guar strains are marked with bold (the Neighbor-Joining method).

The analysis of the ITS-region sequence is often used to additionally identify microorganisms [11, 19]. Comparison of ITS-region showed that all isolates clustered together with a typical strain *B. elkanii* USDA 76T at 100 % statistical support level (Fig. 3). The maximum homology of ITS-region was between thee new isolates and typical strains *B. elkanii, B. tropiciagri* and *B. pachyrhizi*, 94.3, 92.1 and 90.8 %, respectively (see Table).

The name of the species *Bradyrhizobium elkanii* was offered in 1992 [20] for a homologous group of strains within the existing species *B. japonicum*, described for nitrogen-fixing soya microsymbionts *Glycine max* [21]. Both species belong to a predominant genus capable to nodule the majority of varieties of the tribe *Genisteae* [22]; the most known representatives are lupine (*Lupinus*), broom

(*Cytisus*) and greenweed (*Genista*). However, the strains *B. elkanii* were also discovered in nodules of leguminous plants from the tribe *Indigofereae*, which guar belongs to, i.e. in cow pea (*Vigna unguiculata*, *V. radiate*), rosewood (*Dalbergia odorifera*) and beggarweed (*Desmodium incanum*) [23-25]. Earlier on, two isolates from *Cyamopsis*, XBD2 SARCC-388 and ENNRI 16A) capable of effective nitrogen fixation and identified as *B. japonicum* and *Bradyrhizobium* sp., respcetiely, were described [26, 27]. However, the guar was primarily studied for enriching the natural gum properties [28], resistance to illnesses and selection of highly productive varieties [2, 29]. Probably, it is due to no necessity of inoculation because of the sufficient indigenous microsymbionts in places of traditional cultivation of the crop. However, introduction of guar to new habitats makes the plant-microbe symbiosis, which provides plants with nitrogen nutrition, the major factor to effectively cultivate this crop.

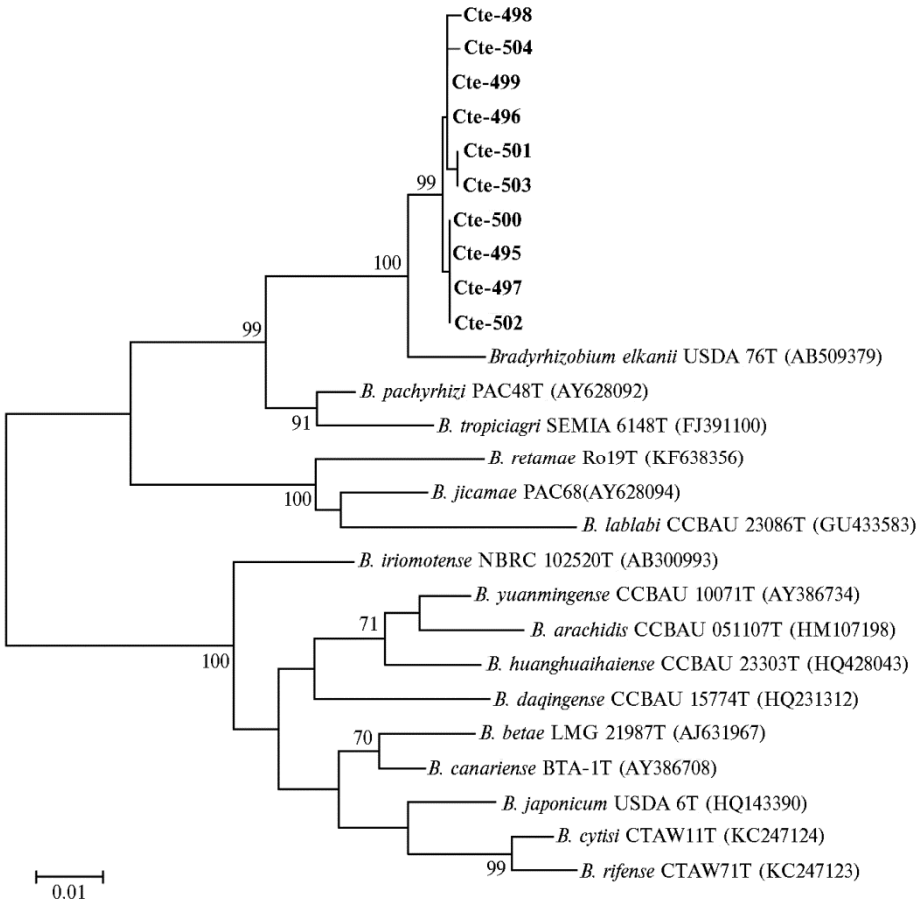

Fig. 3. ITS-phylogram of isolates from guar *Cyamopsis tetragonoloba* nodules and the members of related *Bradyrhizobium* species. Typical strains are marked with the letter T. Guar strains are marked with bold (the Neighbor-Joining method).

Thus, by sequencing *rrs* gene, ten strains isolated from guar root nodules in pot tests, are referred to the species *Bradyrhizobium*. Additional identification (sequencing the ITS-region and three "housekeeping" genes, *dnaK, recA* and atpD), allowed us to specify the taxonomic position of isolates and to show their relation to species *Bradyrhizobium elkanii*. Earlier members of this species were not known as microsymbionts of *Cyamopsis tetragonoloba*. The sequencing of ITS-region and "housekeeping" genes show genetic heterogeneity of natural population of guar microsymbionts that can testify to distinctions in symbiotic

mutual relations between plants and the isolated strains. Further study of genetic diversity, morphological, physiological, biochemical features and economically useful traits of the guar nodule bacteria will allow us to widen knowledge about phylogeny of microsymbionts of this crop which is rather new to Russia and also to select strains effectively improving plant growth and quality of production in new regions of cultivation.

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