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ARAŞTIRMA MAKALESİ RESEARCH PAPER

Diversity of *Mesorhizobium* Species Nodulating Some Wild Legumes in Samsun Province of Turkey

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Abstract: In this study, microsymbionts of two wild legumes, *Argyrolobium biebersteinii* (Ball in Feddes) and *Lotus angustissimus* L., collected from Samsun province of Turkey were investigated using conventional and molecular methods. A total of five rhizobial isolates which produced active root nodules on their original hosts were considered. Identifications of the isolates were depending on the phylogenetic analyses of two housekeeping genes, 16S rDNA and *recA*. As a result of phylogenetic analyses, all isolates appeared within *Mesorhizobium* lineage. Of these isolates, OKN-1 and OKN-4 identified as *Mesorhizobium tarimense* and OKN-3 identified as *M. japonicum*. On the other hand, other two isolates, OKN-7 and OKN-10 did not match up with any known *Mesorhizobium* species. In this study we present the first *Mesorhizobium* isolates identified using valid molecular methods from Turkey. We also give the first reports of *M. tarimense* and *M. japonicum* from Turkey and Europe. Additionally, the new *M. tarimense* and *M. japonicum* isolates in this study are the first isolates reported after the description of these two species from their original hosts and locations. In this study we also present molecular evidences for two new *Mesorhizobium* species, but this presumption needs further investigations.

Keywords: 16S rDNA, *Mesorhizobium*, phylogeny, *recA*, wild legumes..

Türkiye'nin Samsun İlindeki Bazı Yabani Baklagil Türlerini Nodüle Eden *Mesorhizobium* Türlerinin Çeşitliliği

Öz: Bu çalışmada Türkiye'nin Samsun ilinden toplanan iki yabani Baklagil türünün (*Argyrolobium biebersteinii* (Ball in Feddes) and *Lotus angustissimus* L.,) mikrosimbiyontları geleneksel ve moleküler yöntemler kullanılarak incelenmiştir. Toplamda orijinal konakçıları üzerinde aktif kök nodülleri oluşturan beş adet rizobiyal izolat incelenmiştir. Bu izolatların teşhisleri 16S rDNA ve *recA* olmak üzere iki genin filogenetik analizleri ile yapılmıştır. Filogenetik analizler sonucunda tüm izolatlar *Mesorhizobium* soy hattı içerisinde yer almıştır. Bu izolatlardan OKN-1 ve OKN-4 *Mesorhizobium tarimense* ve OKN-3 *M. japonicum* olarak teşhis edilmişlerdir. Diğer taraftan diğer iki izolatımız OKN-7 ve OKN-10, bilinen hiçbir *Mesorhizobium* türü ile eşleşmemiştir. Sonuç olarak bu çalışmada Türkiye'den *Mesorhizobium* cinsine ait ilk moleküler olarak tanımlanmış örnekleri sunmaktayız. Ayrıca *M. tarimense* ve *M. japonicum* türleri için Türkiye'den ve Avrupa'dan ilk kayıtları da vermekteyiz. Ek olarak bu çalışmadaki yeni *M. tarimense* ve *M. japonicum* izolatları, bu iki türün orijinal konaklarından ve lokalitelerinden tanımlanmasından sonra bildirilen ilk izolatlardır. Bu çalışmada iki yeni *Mesorhizobium* türü için de moleküler kanıtlar sunuyoruz, fakat bu ön görünün daha fazla araştırılması gerekmektedir.

Anahtar sözcükler: 16S rDNA, filogeni, *mesorhizobium*, *recA*, yabani baklagiller.

INTRODUCTION

Although 80% of earth's atmosphere composed of nitrogen gas (N_2), this form of nitrogen cannot be directly used by most of the living organisms to synthesis nitrogen containing organic compounds. Initially, nitrogen gas should be reduced to ammonia (biologically available nitrogen) by combining it with hydrogen and this process is called nitrogen fixation (Burdass, 2002). Biological reduction of nitrogen gas to ammonia, so called biological nitrogen fixation, is restricted to prokaryotic species which are distributed across both Bacteria and Archaea domains and provides about 65% of biosphere's available nitrogen (Lodwig et al., 2003; Raymond et al., 2004). Some of these prokaryotes can only able to fix nitrogen when they are in a symbiotic associations with their particular hosts and that is why they have been named as symbiotic diazotrophs (Sawada et al., 2003). Rhizobia, one of the best known symbiotic diazotrophic microorganisms, are soil bacteria that form root or stem nodules on leguminous plants, where they can undertake symbiotic fixation of atmospheric nitrogen (Moreira et al., 1998). Until 1980s, all root nodulating symbiotic diazotrophic bacteria were classified within a single genus namely *Rhizobium* with six species, *R. leguminosarum*, *R. phaseoli*, *R. trifolii*, *R. meliloti*, *R. lupini*, *R. japonicum*, depending on specific host plant selection which was also called cross inoculation group concept (Somasegaran & Hoben, 1985). On the other hand, this concept was abandoned because it is understood that in fast-growing rhizobia, the genes coding for symbiotic associations are located on giant transmissible plasmids called symbiotic plasmids (Brewin et al., 1980). During this period, bacterial systematics underwent a huge change with the impressive contribution of molecular biology. Rhizobial systematics were also affected from this change and eventually species and genus limitations were reassessed due to polyphasic approach which considers molecular data (DNA-DNA hybridization, phylogenetic analyses depending on the nucleotide sequences of different genes) as well as phenotypic features (Graham et al., 1991). In the course of time, numbers of rhizobial species and genera have dramatically increased due to these changes in rhizobial systematics and also to the investigation of more legume species in the meaning of their sysmbiotic partners (Willems, 2006). Currently rhizobia are classified within 238 species (18 genera) of α- and β-Proteobacteria classes and this number is expected to increase since only 23% of legume species (roughly 19000 species exists) were investigated in the context of their symbiotic partners (Shamseldin et al., 2017). *Mesorhizobium*, the main objective of the study, was suggested as a new genus by Jarvis et al., (1997) depending on the differences in 16S rDNA sequences and also fatty acid profiles. Researchers classified five species, *M. cicer*, *M. huakuii*, *M. loti*, *M. mediterraneum* and *M. tianhanense* within this newly suggested genus. Currently, there are 41 *Mesorhizobium* species most of which nodulates mimosoid temperate wild legumes except *M. cicer*, *M. mediterraneum* and *M. muleiense*, which nodulates *Cicer arietinum* L (Sprent, 2007; Shamseldin et al., 2017; http://www.bacterio.net/mesorhizobium.html). From Turkey there are no records for any *Mesorhizobium* species identified using valid molecular methods from cultivated or wild legumes.

The main goal of the study is to identify some rhizobial samples isolated from root nodules of two wild legume species, *Argyrolobium biebersteinii* (Ball in Feddes) and *Lotus angustissimus* L., collected from Samsun province of Turkey.

MATERIAL and METHODS

In this study, rhizobial strains were isolated from root nodules of two wild Leguminous species, *Argyrolobium biebersteinii* (Ball in Feddes) and *Lotus angustissimus* L., collected from Samsun province of Turkey. Bacterial isolations from active (pink colored) root nodules were made using the method of Somasegaran and Hoben, (1985). YMA (Yeast Extract Mannitol Agar) medium was used for isolations. After 2-7 days of incubation at 26°C, typical colonies were re-inoculated on the new YMA medium for further analyses. Purity of the isolates were checked with microscopic examination by gram staining. Nodulation tests were performed using the method of Vincent (1970) with the conditions explained in Gurkanli et al. (2013). For genomic DNA isolations, isolates were grown in TY (Tryptone Yeast Extract) broth media (Ditta et al. 1987) at 28°C for 2 days. The CTAB/NaCl miniprep method (Maniatis et al. 1982; Temizkan and Arda 2004) was used for genomic DNA extractions and the DNA were stored at -20°C prior to use. Two housekeeping genes, 16S rDNA (Small subunit of rDNA) and *recA* (Recombinase A) were analysed for identification of rhizobial isolates. PCR amplifications of the genes were performed using a MWG Primus thermal cycler with the protocols and DNA primers shown in Table 1. A 50 µl PCR mixture for all genes were prepared as follows: 5 µl of 10X PCR buffer (Fermentas), 1.5 mM $MgCl_2$, 0.8 mM dNTP mix (Amresco), 0.4 pmol of each primer in final concentration, 1.25 U Taq polymerase (Fermentas), template DNA<0.5 µg and ddH_2O. Visualization of the PCR products which were electrophoresed on 1% agarose gel (prepared in 1X Tris-Borate-EDTA buffer and stained with ethidium bromide) were made using the GeneGenius Bio imaging system (Syngene, Synoptics Group, Cambridge, UK). Nucleotide sequencings were performed from both strands with the primers used for the PCR amplifications (Table 1). The only exception was an extra 16S rDNA internal primer pF (Zhang et al., 1999) that we used for more reliable sequencings.

Table 1. Nucleotide primers and PCR conditions used for the PCR amplifications of 16S rDNA and *recA* genes in this study. [A](Weisburg et al., 1991); [B](Gaunt et al., 2001).

Gene	Primer	ID	C	D	A	E	FE
16S rDNA	fD1/rD1[A]	95°C / 3 min	× 35	95°C / 1 min	55°C / 1 min	72°C / 1.5 min	72°C / 5 min
recA	recA-Forward/ recA-Reverse[B]	95°C / 5 min	× 35	95°C / 45 sec	50°C / 1 min	72°C / 1 min	72°C / 2 min

ID: Initial denaturation; **C:** Cycle; **D:** Denaturation; **A:** Annealing; **E:** Elongation; **FE:** Final elongation

The nucleotide sequencings were made commercially by Macrogen Inc. (Korea). Nucleotide sequencings from both strand were checked and assembled using BioEdit (Hall, 1999). ClustalX (Thompson et al., 1997) was employed for multiple nucleotide sequence alignments. To determine the most appropriate DNA substitution model for our data sets, the Akaike information criterion (AIC) (Akaike, 1974) and Bayesian information criterion (BIC) tests were applied with jModelTest v. 0.1 package program (Guindon & Gascuel, 2003; Posada, 2008). Initial phylogenetic analyses were performed with extended data sets comprised of 16S rDNA and *recA* haplotypes of all valid *Mesorhizobium* species.

Table 2: Strain informations of *Mesorhizobium* isolates obtained in this study and the type strains of *Mesorhizobium* species download from NCBI for phylogenetic analyses.

Species	*Strain*	*16S rDNA*	*recA*
M. tarimense	OKN-1	MN647524 / This study	MN658186 / This study
M. japonicum	OKN-3	MN647525 / This study	MN658187 / This study
M. tarimense	OKN-4	MN647526 / This study	MN658188 / This study
Mesorhizobium spp.	OKN-7	MN647527 / This study	MN658189 / This study
Mesorhizobium spp.	OKN-10	MN647528 / This study	MN658190 / This study
M. amorphae	ACCC 19665	-	AY688612 / Vinuesa et al., (2005)
M. australicum	WSM2073	AY601516 / Nandasena et al., (2009)	JN202310 / Zhang et al. Unpublished
M. cantuariense	ICMP 19515	KC237397 / De Meyer et al., (2015)	KC237677 / De Meyer et al., (2015)
M. caraganae	CCBAU 11299	EF149003 / Guan et al., (2008)	EU249394 / Guan et al., (2008)
M. cicer	UPM-Ca	U07934 / Nour et al., (1994)	KC237677 / Gaunt et al., (2001)
M. erdmanii	USDA 3471	KM192334 / Martinez-Hidalgo et al., (2015)	-
M. gobiense	CCBAU 83330	EF035064 / Han et al., (2008)	EF549481 / Han et al. unpublished
M. helmanticense	CSLC115N	Sannazzaro et al. unpublished	Sannazzaro et al. unpublished
M. huakuii	CCBAU 260	D13431 / Oyaizu et al., (1993)	EU249391 / Guan et al., (2008)
M. japonicum	MAFF303099	NC_002678/ Martinez-Hidalgo et al., (2016)	NC_002678/ Martinez-Hidalgo et al., (2016)
M. jarvisii	ATCC 33669	KM192335 / Martinez-Hidalgo et al., (2015)	KM192345 / Martinez-Hidalgo et al., (2015)
M. loti	NZP 2213	X67229 / Willems et al., (1993)	EU039875 / Alexandre et al., (2008)
M. metallidurans	STM 2683	AM930381 / Vidal et al., (2009)	AM930382 / Vidal et al., (2009)
M. muleiense	CCBAU 83963	-	HQ316782 /Zhang et al., (2012)
M. opportunistum	WSM2075	AY601515 / Nandasena et al., (2006)	-
M. qingshengii	CCBAU 33460	JQ339778 / Zheng et al., (2013)	JQ339757 / Zheng et al., (2013)
M. septentrionale	SDW014	-	EF639843 / Han Unpublished
M. shangrilense	CCBAU 65327	EU074203 / Lu et al., (2009)	-
M. tarimense	CCBAU 83306	EF035058 / Han et al., (2008)	EF549482 / Han Unpublished
M. tianshanense	A-1BS	AF041447 / Wang et al., (1999)	EU249392 / Guan et al., (2008)
M. waimense	ICMP 19557	KC237387 / De Meyer et al., (2015)	KC237667 / De Meyer et al., (2015)

Subsequently, more detailed analyses were conducted with concentrated data sets only composed of closely related *Mesorhizobium* species with our haplotypes (Table 2). Neighbor-Joining (NJ), Maximum-Likelihood (ML) and Maximum-Parsimony (MP) analyses were performed to evaluate the phylogenetic relationships. PAUP* v. 4.0b10 (Swofford, 1998) was used for the NJ and MP analyses and PhyML 3.0 (Guindon & Gascuel, 2003) was used for ML analysis. MP analysis were performed with the heuristic search approach by using the TBR swapping algorithm (10 random repetitions). To determine the reliability of the trees, the Bootstrap tests were performed with 10000 pseudo replicates for NJ and 1000 pseudo replicates for MP and ML. All our new 16S rDNA and *recA* sequences obtained in this study were deposited in GenBank under accession numbers MN647524-MN647528 and MN658186-MN658190, respectively (Table 2).

RESULTS

As the result of bacterial isolations, a total of 5 rhizobial samples were obtained from root nodules of *Argyrolobium biebersteinii* P.W. Ball (OKN-1, OKN-4, OKN-7, OKN-10) and *Lotus angustissimus* L. (OKN-3). The isolates formed active (pink colored) root nodules on their original hosts. To identify our isolates, we analysed two housekeeping genes, 16S rDNA and *recA*. We sequenced approximately 1315 bp of 16S rDNA of our isolates and BLAST (Basic Local Alignment Search Tool) results indicated that all our samples belonged to the genus *Mesorhizobium*. Phylogenetic analyses were performed with 1258 aligned nucleotides containing 76 segregating sites. AIC and BIC tests suggested GTR+I+G (I=0.838; G=0.785) and TPM3uf+I (I=0.904) substitution models, respectively. Since it gave higher bootstrap values we preferred the NJ tree drawn with TPM3uf+I model (Figure 1). As a result, our isolates, OKN-1 and OKN-4 from *A. biebersteinii* showed the same 16S rDNA haplotype with each other and also with *M. tarimense*, *M. gobiense*, *M. helmanticense* and *M. metallidurans* and formed a lineage with *M. tianshanense*. Our other isolate OKN-7, also from *A. biebersteinii*, showed a unique 16S rDNA haplotye and appeared as sister to *M. caraganae* with 99.7% nucleotide sequence similarity. This

relationship was supported with 62%, 63% and 61% bootstrap values in NJ, ML and MP trees, respectively.

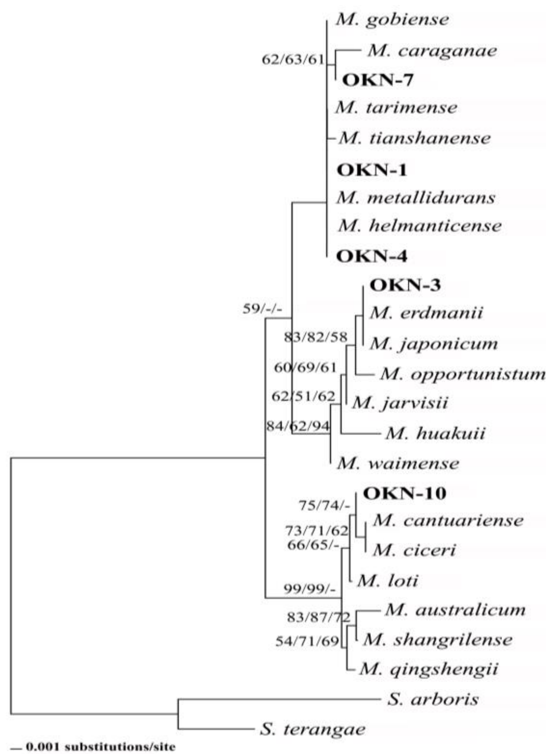

Figure 1. NJ tree based on the 16S rDNA nucleotide sequences of our new *Mesorhizobium* isolates (Bold) and the type strains of closely related *Mesorhizobium* species downloaded from GenBank (Table 2). The tree was rooted with *Sinorhizobium arboris* (Zhang et al., 1991) and *S. terangae* (de Lajudie, 1994). Bootstrap values (≥ 50%) obtained from NJ, ML and MP analyses are given on each related node.

OKN-10, our fourth isolate from *A. biebersteinii*, appeared as sister to *M. cantuariense* and *M. cicer* (showed the same 16S rDNA haplotypes) with 99.9% nucleotide sequence similarity and this node was supported with 75% and 74% bootstrap values in NJ and ML trees, respectively. On the other hand, this value was less than 50% in MP tree. *M. loti* also appeared as the fourth species in the lineage. Although this lineage was quite consistent and appeared in all trees created with NJ, ML and MP algorithms, intralineage relationships were different. In NJ and ML trees, topology were the same as explained above and all nodes in this lineage were supported with significant bootstrap values. On the other hand, a polytomy appeared in the MP bootstrap tree, thus some bootstrap values were less than 50%. Isolate OKN-3, from *L. angustissimus*, showed the same 16S rDNA haplotype with *M. japonicum* and *M. erdmanii*. *M. opportunistum* appeared as sister with 99.7% nucleotide similarity. This relationship supported with 83%, 82% and 58% bootstrap values in NJ, ML and MP trees, respectively. *M. jarvisii*, *M. huakuii* and *M. waimense* also appeared in the lineage (Figure 1).

As the second housekeeping gene we sequenced approximately 570 bp of *recA* gene of our isolates. Phylogenetic analyses were carried over 317 aligned nucleotides with 98 segregating sites. AIC and BIC tests were suggested TIM2+I+G (I=0.383; G=0.388) and TrN+G (G=0.172) substitution models, respectively. Since the tree created with TIM2+I+G model gave higher bootstrap values, this tree preferred in the study (Figure 2). In general, *recA* phylogeny was concordant with the 16S rDNA phylogeny with minor differences. Isolates OKN-1 and OKN-4 showed close *recA* haplotypes with *M. tarimense* with 99.6% nucleotide similarities and this relationship supported with 99%, 99% and 93% bootstrap values in the NJ, ML and MP trees, respectively. *M. gobiense*, *M. muleiense* and *M. tianshanense* also appeared in this lineage (Figure 2). Phylogenetic position of OKN-7 in *recA* phylogeny was quite different than 16S rDNA phylogeny. In the *recA* tree (Figure 2), this isolate (together with *M. caraganae*) appeared as sister to *M. metallidurans*, *M. septentrionale*, *M. waimense*, *M. amorphae* and *M. helmanticense* with 94.9%, 95.5%, 95.8%, 95.2%, 96.2% nucleotide sequence similarities, respectively. On the other hand, this lineage was not supported with enough (≥50) bootstrap values.

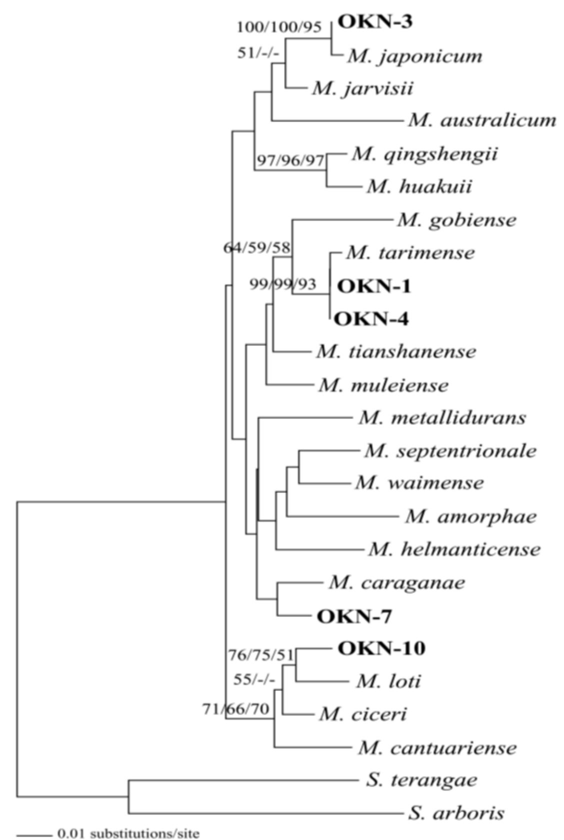

Figure 2. NJ tree based on the *recA* nucleotide sequences of our new *Mesorhizobium* isolates (Bold) and the type strains of closely related *Mesorhizobium* species downloaded from GenBank (Table 2). The tree was rooted with *Sinorhizobium arboris* (Lloret et al., 2007) and *S. terangae* (Martens et al., 2007). Bootstrap values (≥ 50%) obtained from NJ, ML and MP analyses are given on each related node.

Our other isolate OKN-10 appeared as sister to *M. loti* with 97.4% nucleotide sequence similarity and this relationship was supported with 76%, 75% and 51% bootstrap values in the NJ, ML and MP trees, respectively. Concordant with the phylogeny of 16S rDNA, *M. ciceri* and

M. cantuariense also appeared as related to the lineage above and this lineage was supported with bootstrap values higher than 50% in NJ and ML trees. OKN-3 revealed *recA* haplotype close to *M. japonicum* with 99.6% nucleotide sequence similarity and this relationship was supported with 100%, 100% and 95% bootstrap values in the NJ, ML and MP trees, respectively. Additionally *M. australicum*, *M. jarvisii*, *M. qingshengii* and *M. huakuii* also appeared in the same lineage. On the other hand, *M. erdmanii* and *M. opportunistum* which were closely related to this isolate in 16S rDNA phylogeny (Figure 1) did not appeared in the lineage in *recA* phylogeny.

DISCUSSION

Symbiotic diazotrophic bacteria commonly known as rhizobia is a popular subject for scientists all over the world because of their ecological and economical importance. So far some studies concerning rhizobia from Turkey have been published, however most of them are related to agronomic applications of rhizobial isolates (İçgen et al. 2002; Tüfenkci et al. 2006; Küçük & Kıvanç, 2008a; Togay et al. 2008). On the other hand, only a couple of studies about the diversity of rhizobia in Turkey are available and the isolates in these studies only belong to genus *Rhizobium* (Küçük et al. 2006; Küçük & Kıvanç, 2008b; Öğütçü et al. 2008; Ogutcu et al., 2009; Adiguzel et al., 2010; Gurkanli et al., 2013, 2014; Canık Orel et al. 2016). In this study, we characterized 5 rhizobial samples, OKN-1, -3, -4, -7, -10, isolated from root nodules of two wild legumes, *Argyrolobium biebersteinii* (Ball in Feddes) and *Lotus angustissimus* L., collected from Samsun province of Turkey. Phylogenetic analyses depending on 16S rDNA (Figure 1) clearly indicated the relationships of these isolates with the genus *Mesorhizobium*. From Turkey there are no records for any *Mesorhizobium* isolates identified using valid molecular methods, thus these isolates are the first ones. On the other hand, 16S rDNA did not provide sufficient information to associate our isolates with one of the valid *Mesorhizobium* species (Figure 1). Condordant with our result, recent studies have clearly indicated that phylogenetic analysis solely depending on 16S rDNA do not fully resolve the evolutionary relationships among rhizobial isolates in species level due to the high degree of conservation of the gene (Mousavi et al., 2015). That's why, we used a second housekeeping gene, *recA*, to identify our isolates. As a result, although isolates OKN-1 and OKN-4 from *A. biebersteinii* showed the same 16S rDNA haplotype with *M. tarimense*, *M. gobiense* and *M. metallidurans* (Figure 1), *recA* phylogeny clearly revealed their relationship with *M. tarimense* (Figure 2) which was originally identified from root nodules of *Lotus* sp. in Xinjiang, China (Han et al., 2008). To our knowledge, OKN-1 and OKN-4 are the only *M. tarimense* isolates reported after its description from China. Similarly, isolate OKN-3 from *L. angustissimus* L. showed the same 16S rDNA haplotype with *M. erdmanii* and *M. japonicum* (Figure 1), on the other hand in *recA* phylogeny, this isolate appeared as closely related to the later species (Figure 2). *M. japonicum* was identified with reclassification of two *Lotus* sp. nodulating bacteria from Japan and New Zealand which were formerly identified as *M. loti* (Martinez-Hidalgo et al., 2016). Since then, this species have not been reported from else where, thus this is the first report of *M. japonicum* from Turkey and Europe. Although, isolate OKN-10 from *A. biebersteinii* placed in the same lineage with *M. loti*, *M. cicer* and *M. cantuariense* in 16S rDNA (Figure 1) and *recA* trees (Figure 2), it did not showed enough similarity with none of these species to designate it to one of them, thus it is possible that this isolate is a new species. Likewise, OKN-7 probably represents a new *Mesorhizobium* species, since we could not designate this isolate to any of the available *Mesorhizobium* species according to 16S rDNA and *recA* phylogenies. These presumptions needs further investigations.

In conclusion, this study presents the first *Mesorhizobium* isolates identified using valid molecular methods from Turkey. Additionally, first reports of *M. tarimense* and *M. japonicum* from Turkey and Europe are also given in the study. These isolates are the first *M. tarimense* and *M. japonicum* isolates reported after the description of these two species from their original hosts and locations. This study also presents molecular evidences for two new *Mesorhizobium* species, but these presumptions needs further investigations.

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