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Molecular identifications of rhizobial samples isolated from Phaseolus vulgaris L. in Eskişehir province of Turkey

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Abstract

In this study three rhizobial samples (R28, R51, R66) were anaysed using molecular phylogenetic methods. Rhizobial samples were isolated (from *P. vulgaris* L. root nodules collected from Eskişehir province of Turkey) and morphologically characterized in a previous study. Analyses were performed using a concatenated data set which composed of three housekeeping genes (*recA, atpD* and *glnII*). Additionally two symbiotic genes, *nodA* and *nifH*, were sequenced and analysed to determine the symbiotic plasmid type. As a result of housekeeping gene phylogeny, isolate R28 was found to be related to *R. laguerreae*. Although other two isolates, R51 and R66, placed in the same lineage with *R. sophoriradicis*, they showed enough divergence to be considered as a new species. But this presumption need to be confirmed with further investigations. Network analyses of *nodA* and *nifH* genes clearly showed that R28 has a unique symbiotic plasmid. On the other hand, symbiotic plasmids of R51 and R66 found to be related to p42d which is the most common symbiotic plasmid in rhizobia nodulating *P. vulgaris* L. in Europe and also Turkey. In this study first report of *R. laguerreae* from Turkey is presented. More over, first report of a *R. laguerreae* isolate from *P. vulgaris* L. root nodules is also presented and suggested isolate R28 as *R. laguerreae* bv. *phaseoli*. Additionally, molecular hints for a potentially new rhizobial species have also given. But this presumption must be supported with some additional molecular and morphological investigations.

Key words: rhizobia, phylogeny, housekeeping genes, nodulation, nitrogen fixation

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Türkiye'nin Eskişehir ilinden, *Phaseolus vulgaris* L.'den izole edilen rhizobial örneklerin moleküler karakterizasyonu

Özet

Bu çalışmada üç rizobial örnek (R28, R51, R66) moleküler filogenetik yöntemler kullanarak analiz edilmiştir. Rizobial örnekler önceki bir çalışmada izole edilmiş (Türkiye'nin Eskişehir ilinden toplanmış *P. vulgaris* L. bitkisinin kök nodüllerinden) ve morfolojik analizleri yapılmıştır. Analizler üç adet housekeeping genden (*recA, atpD* and *glnII*) oluşan birleştirilmiş bir veri seti kullanılarak yapılmıştır. Ek olarak, simbiyotik plazmit tipini belirlemek için *nodA* ve *nifH* olmak üzere iki simbiyotik genin dizilemesi yapılmış ve analizleri gerçekleştirilmiştir. Housekeeping gen filogenisin sonucu olarak izolat R28 *R. laguerreae* ile ilişkili olarak bulunmuştur. Diğer iki izolat R51 ve R66, *R. sophoriradicis* ile aynı soy hattında yer almasına rağmen yeni bir tür sayılabilecek kadar yeterli farklılık göstermiştir. Fakat bu öngörünün ileri araştırmalar ile doğrulanmaya gereksinimi vardır. *NodA* and *nifH* genlerinin network analizleri R28 in özgün bir simbiyotik plazmite sahip olduğunu açık bir şekilde göstermiştir. Diğer taraftan R51 ve R66'nın simbiyotik plazmitlerinin p42d ile ilişkili olduğu bulunmuştur, bu plazmit Avrupa ve Türkiye'de *P. vulgaris* L. ile kök nodülü oluşturan rhizobiadaki yaygın görülen simbiyotik plazmittir. Bu çalışmada *R. laguerreae* in Türkiye'den ilk kaydı sunulmuştur. Ötesinde, bir *R. laguerreae* izolatının *P. vulgaris* L. kök nodüllerinden ilk kaydıda verilmiştir ve izolat R28 *R. laguerreae* bv. *phaseoli* olarak önerilmiştir. Ek olarak yeni bir rizobial tür için moleküler ip uçlarıda sunulmuştur. Fakat bu öngörünün ilave bazı moleküler ve morfolojik araştırmalar ile desteklenmesi gerekmektedir.

Anahtar kelimeler: rhizobia, filogeni, housekeeping genler, nodülasyon, azot fiksasyonu.

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Introduction

Phaseolus vulgaris L. is the most important food legume in the World which represents 50% of leguminous grain products for direct human consumption (Jones, 1999; Wang et al., 2016). This plant species has two centres of origins, one in Mesoamerica and another in the Andean South America, likewise it also have been domesticated in the same regions for more than 7000 years (Gepts and Bliss 1988; Gepts et al. 1990). Seeds of *P. vulgaris* L. introduced to Anatolia approximately 250-300 years ago and currently is the third commonest leguminous grain in Turkey with 215.000 tons of annual production after chickpeas and lentils (Şehrali, 1988; Gülümser, 2016). Like other leguminous plants, *P. vulgaris* L. roots make symbiotic association with symbiotic diazotrophic bacteria collectively known as rhizobia. Altough *Rhizobium etli* and *R. tropici* are distributed world wide and are dominant symbionts of *P. vulgaris* L., many other local isolates of different rhizobial species have also reported as symbiotic partners because of the promiscuous nature of this plant as a host (Martinez-Romero et al., 1991; Segovia et al., 1993; Tamimi and Young, 2004; Kaschuck et al., 2006). These species includes; *Rhizobium giardinii*, *R. gallicum* (Amarger et al., 1997), *R. lusitanum* (Valverde et al., 2006), *R. phaseoli* (Ramirez-Bahena et al., 2008), *R. azibense* (Mnasri et al., 2014), *R. freirei* (Dall'Agnol et al. 2013), *R. esperanzae* (Cordeiro et al. 2017), *Ensifer meliloti* (Zurdo-Pineiro et al. 2009), *E. americanus* (Mnasri et al., 2012), *E. fredii* like isolates (Herrera-Cervera et al., 1999), *Bradyrhizobium* sp. (Han et al., 2005), *Mesorhizobium* isolates (Grange et al., 2004), *Burkholderia caballeronis* (Martinez-Aguilar et al., 2013). Although molecular based studies about the diversity of various baterial groups from Turkey are available, unfortunately there are not much studies concerning species diversity of rhizobia nodulating *P. vulgaris* L. in Turkey (Bekler, 2016). Most comprehensive study was published by Gurkanli et al. (2013) where *R. leguminosarum* bv. *phaseoli*, *R. etli* bv. *phaseoli* and *R. phaseoli* were reported as symbionts of *P. vulgaris* L. cultivated in Blacksea region of Turkey. Thus, many more molecular based studies are necessary to fully determine the species diversity of rhizobia nodulating *P. vulgaris* L. cultivated in different geographical parts Turkey.

The goal of this study is to phylogenetically examine three rhizobial isolates which were obtained (from *P. vulgaris* L. root nodules collected from Eskişehir province of Turkey) and morphologically characterized in a previous study (Küçük et al., 2006).

2. Materials and methods

For phylogenetic analyses, three morphologically characterized (Küçük et al., 2006) rhizobium isolates (R28, R51 and R66) were selected. The selection was made within the isolates which showed the highest ecological valence. Rhizobium isolates were grown in TY (Tryptone Yeast Extract) broth medium for genomic DNA isolations with the conditions given in Gurkanli et al. (2013). A CTAB/NaCl miniprep method was used for genomic DNA isolations (Temizkan and Arda, 2004). For identification of isolates three housekeeping genes, *recA* (recombinase A), *atpD* (ATP synthase subunit beta), *glnII* (glutamine synthetase II) were analysed. Additionally, two symbiotic genes, *nodA* (acyltransferase nodulation protein) and *nifH* (nitrogenase reductase Fe protein) were analysed for determination of the sym plasmid (symbiotic plasmid) type. All PCR amplifications were made with the primers and PCR conditions explained in Gurkanli et al. (2013). PCR amplifications were performed using a Techne (TC-Plus) thermal cycler and products were electrophoresed on 1% agarose gel (Amresco, USA) which prepared in 1X TBE. A Vilber Lourmat Imaging System was used to visualize the PCR products. Nucleotide sequencings were made commercially by Macrogen (Korea) from both strands with the same primers used for the PCR amplifiations. BioEdit (Hall 1999) was used to assemble the sequencings from both strands. Multiple nucleotide sequence alignments were generated using ClustalX (Thompson et al., 1997) and optimized by hand using BioEdit. Akaike information criterion (AIC; Akaike, 1974) and Bayesian information criterion (BIC) tests were performed using jModelTest v. 0.1 package program (Guindon and Gascuel, 2003; Posada, 2008) to determine the best fitting evolutionary model(s) to our data sets. To evaluate the phylogenetic relationships between haplotypes, Neighbor-Joining (NJ), Maximum-Parsimony (MP) and Maximum-Likelihood (ML) algorithms were employed. NJ and MP analyses were performed using PAUP* v. 4.0b10 (Swofford, 1998) and PhyML 3.0 (Guindon and Gascuel, 2003) was used for ML analyses. MP analyses were performed with the heuristic search approach by using the TBR swapping algorithm (10 random repetitions). 10 000 pseudo-replicates were conducted for the Bootstrap tests (Efron, 1982; Felsenstein, 1985) of the NJ analyses and 1,000 pseudo-replicates for MP and ML analyses. For symbiotic genes we conducted Neighbor NetWork analyses using the Splitstree4 program (Huson and Bryant, 2006). All our new sequences have deposit in GenBank under accession numbers MH299832 MH299846 (Table).

3. Results

To identify our isolates (R28, R51 and R66) we sequenced approximately 570, 500 and 650 bp of their three housekeeping genes *recA*, *atpD* and *glnII*, respectively. To construct phylogenies we created a concatenated sequence data with these genes.

Table. Names, GenBank accession numbers and sources of rhizobia used in this study

Species	**Isolate**	**GenBank Accession Numbers**			**Source**
		recA	***atpD***	***glnII***	
Rhizobium spp.	R28	MH299835	MH299838	MH299832	This study
Rhizobium spp.	R51	MH299836	MH299839	MH299833	This study
Rhizobium spp.	R66	MH299837	MH299840	MH299834	This study
R. leguminosarum	USDA 2370^{T}	AJ294376^{A}	AJ294405^{A}	EU155089^{B}	^{A}Gaunt et al. (2001) ^{B}Han et al. (Unpublished)
R. indigoferae	CCBAU 71042^{T}	EF027965^{A}	GU552925^{B}	JN580717^{C}	^{A}Martinez-Romero and Lloret (Unpublished) ^{B}Wu et al. (2011) ^{C}Aserse et al. (2012)
R. sophorae	CCBAU 03386^{T}	KJ831252	KJ831235	KJ831241	Jiao et al. (2015)
R. laguerreae	FB206^{T}	JN558681	JN558661	JN558671	Saidi et al. (2014)
R. laguerreae	FB14022	JN558690	JN558670	JN558680	Saidi et al. (2014)
R. laguerreae	FB310	JN558682	JN558662	JN558672	Saidi et al. (2014)
R. laguerreae	FB403	JN558683	JN558663	JN558673	Saidi et al. (2014)
R. anhuiense	CCBAU 23252^{T}	KF111980	KF111890	KF111913	Zhang et al. (2015)
R. fabae	CCBAU 33202^{T}	EF579941	EF579929	EF579935	Tian et al. (2008)
R. pisi	DSM 30132^{T}	DQ431676^{A}	EF113149^{B}	JN580715^{C}	^{A}Ramirez-Bahena et al. (2008) ^{B}Santillana et al. (2008) ^{C}Aserse et al. (2012)
R. vallis	CCBAU 65647^{T}	GU211770	GU211768	GU211771	Wang et al. (2011)
R. acidisoli	FH13^{T}	KJ921098	KJ921069	KJ921080	Verastegui-Valdes et. al (2014)
R. sophoriradicis	CCBAU 03470	KJ831248	KJ831231	KJ831237	Jiao et al. (2015)
R. sophoriradicis	CCBAU 03433^{T}	KJ831250	KJ831233	KJ831239	Jiao et al. (2015)
R. phaseoli	ATCC 14482^{T}	EF113136^{A}	EF113151^{A}	JN580716^{B}	^{A}Santillana et al. (2008) ^{B}Aserse et al. (2012)
R. bangladeshense	BLR175^{T}	JN649057	JN648967	JN648979	Rashid et al. (2012)
R. etli	USDA 9032	AJ294375^{A}	AJ294404^{A}	AF169585^{B}	^{A}Gaunt et al. (2001) ^{B}Turner and Young (2000)
R. lentis	BLR27^{T}	JN649031	JN648941	JN648976	Rashid et al. (2012)
R. hainanense	CCBAU 57015^{T}	HQ394252	HQ394217	GU726294^{B}	^{A}Robledo et al. (2011) ^{B}Chang et al. (Unpublished)
R. tropici	USDA 9030^{T}	AJ294373^{A}	AJ294397^{A}	AF169584^{B}	^{A}Gaunt et al. (2001) ^{B}Turner and Young (2000)

Our data set was comprised of rhizobium species which showed the highest BLAST scores (Table). Phylogenetic analyses were performed over 1192 aligned nucleotides containing 308 variable sites. Both AIC and BIC tests suggested TIM2+I+G (I: 0.572; G:0.862) substitution model. Parsimony analysis was performed over 225 synapomorphic characters and suggested 3 most parsimonus trees with 713 steps (CI: 0.541374, RI: 649142, HI: 458626). In all phylogenetic trees created with NJ (Figure 1), MP and ML algorithms, our sample R28 appeared in the same lineage with *Rhizobium laguerreae* isolates, FB206^{T}, FB14022, FB310 and FB403. This lineage was supported with sufficient bootstrap values, and the nucleotide sequence similarities among R28 and *R. laguerreae* isolates were between 98.5% and 97.9%. Our other isolates, R51 and R66, showed similar haplotypes with 99.3% nucleotide sequence similarity thus possibly representing same species. This group appeared as sister to *R. sophoriradicis* isolates CCBAU 03470^{T}, CCBAU 03433 and these relationships were supported with very high bootstrap values (Figure 1). Although isolates R51 and R66 formed a lineage with *R. sophoriradicis*, the nucleotide sequence similarities between them were quite low (between 96.3% and 96%) to consider this lineage as a single species.

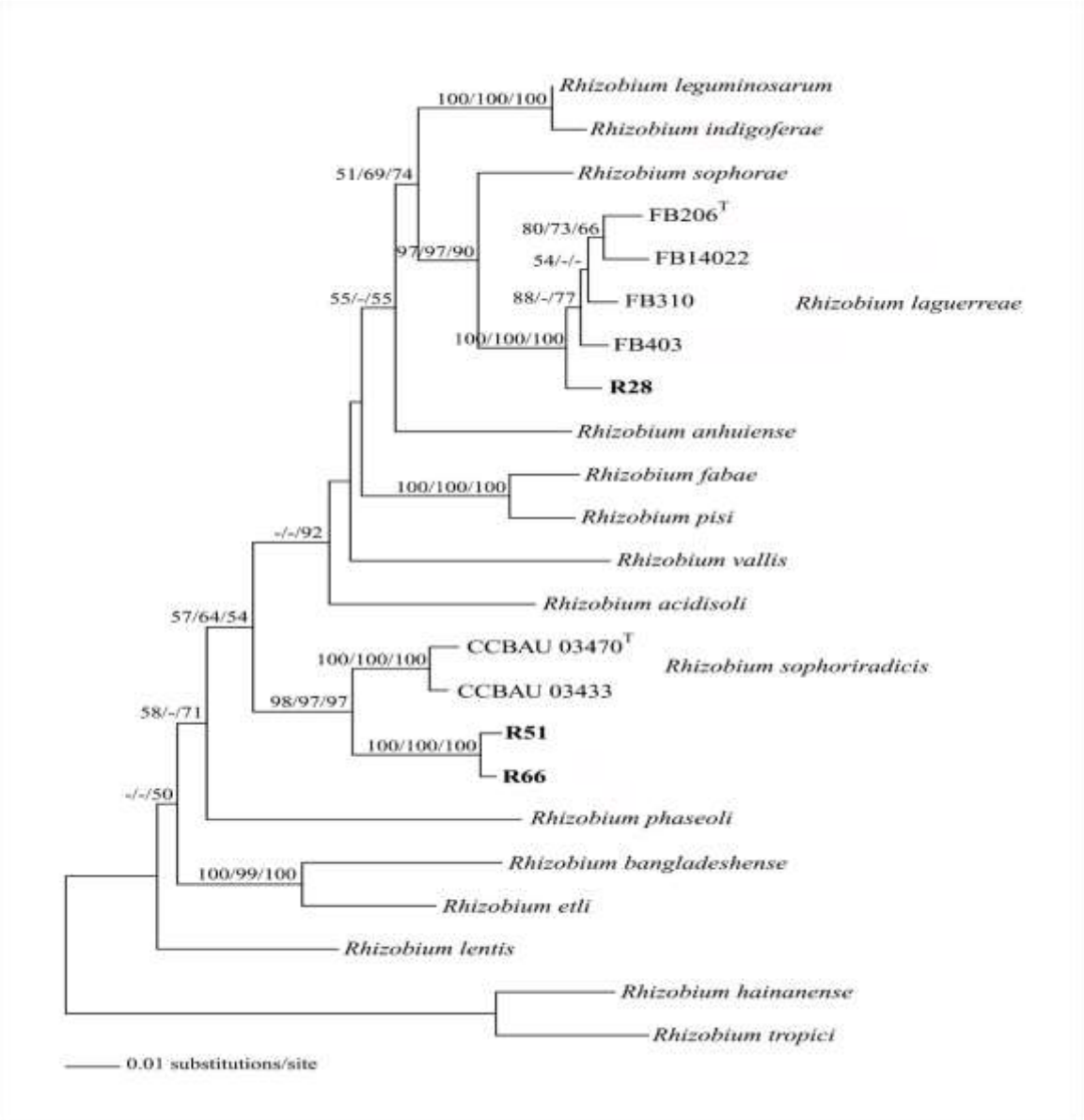

Figure 1. NJ tree showing the phylogenetic relationships between our isolates (R28, R51 and R66) and related *Rhizobium* species abotained from GenBank (Table). The tree is based on concatenated nucleotide sequences of *recA*, *atpD* and *glnII* genes. Bootstap values (≥50%) obtained from NJ, MP and ML analyses are given on each related node

As the representative for the nodulation genes we sequenced approximately 550 bp of the *nodA* (N-acyltransferase) gene to determine the symplasmid type of our isolates. Our data set was comprised of *nodA* haplotypes of different rhizobial species isolated from *P. vulgaris* L. root nodules (Figure 2 legend). A neighbor NetWork analysis was performed over 526 aligned nucleotides containing 262 segregated sites (Figure 2). As a result, our two isolates R51 and R66 showed the same *nodA* haplotype with isolates CTG-403 and CTG-416 which were isolated from the Blacksea part of Turkey in our previous study, Gurkanli et al. (2013). This haplotype appeared as closely related to symbiotic plasmid p42d *nodA* haplotype which was harboured by *R. etli* isolates; CFN-42 and Kim5, *R. phaseoli* isolates; Bra5 and N161, *R. gallicum* bv. *phaseoli* isolate 8C-3 and *R. giardinii* bv. *phaseoli* isolate Ro84 (Figure 2), and the nucleotide sequence similarity between these two haplotypes was 99.8%. On the otherhand our other isolate, R28, showed a unique *nodA* haplotype which is relatively close to *R. gallicum* bv *phaseoli nodA* haplotype with 99.8% nucleotide sequence similarity (Figure 2).

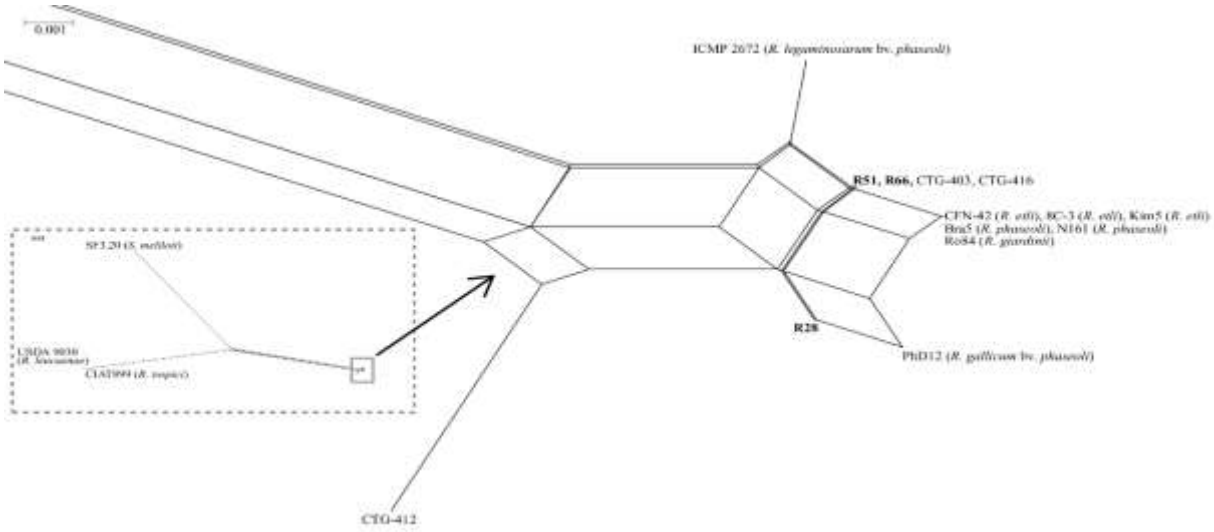

Figure 2. Neighbor network tree derived from *nodA* nucleotide sequences of our isolates, R28 (MH299841), R51 (MH299842), R66 (MH299843) and related *Rhizobium* species obtained from GenBank (Below):
SF3.20_KP765346 (Alias-Villegas et al., Unpublished); CIAT 899_CP004017 (Ormeno-Orrillo et al., 2012); USDA 9039_X98514 (Debelle et al., 1996); Ro84_AJ300239 (Moulin, Unpublished); PhD12_AJ300237 (Moulin, Unpublished); ICMP 2672_DQ100403 (Weir, 2006); CFN 42_U80928 (Girard et al., 1991); Kim5_CP021125 (Santamaría et al., 2017); 8C-3_CP017243 (Bustos et al., 2017); Bra5_CP020898 (Santamaría et al., 2017); N161_CP013587 (Perez-Carrascal et al., Unpublished); CTG-403, CTG-412, CTG-416 (Gurkanli et al., 2013)

As a second type of symbiotic gene we sequenced approximately 750 bp of the *nifH* gene which codes Fe protein of nitrogenase reductase. We conducted a data set containing *nifH* haplotypes of different rhizobial species isolated from *P. vulgaris* L. root nodules. Neighbor NetWork analysis was carried over 456 aligned nucleotides containing 58 variable sites (Figure 3). As a result our isolates R51 and R66 showed the p42d *nifH* haplotype with *R. etli* isolate CFN42, *R. phaseoli* isolates Bra5 and N161, *R. vallis* isolate CCBAU 65647; *R. leguminosarum* bv. *phaseoli* isolate Gut-2; *R. gallicum* bv. *phaseoli* isolate 8C-3 and CTG-403, our previously published isolate (Gurkanli et al., 2013). Another *R. etli* isolate Kim5 showed the closest *nifH* haplotype with 99.3% nucleotide sequence similarity (Figure 3). On the otherhand concordant with *nodA*, isolate R28 showed a unique *nifH* haplotype which is quite close to *R. acidisoli nifH* haplotype with 99.7% nucleotide sequence similarity.

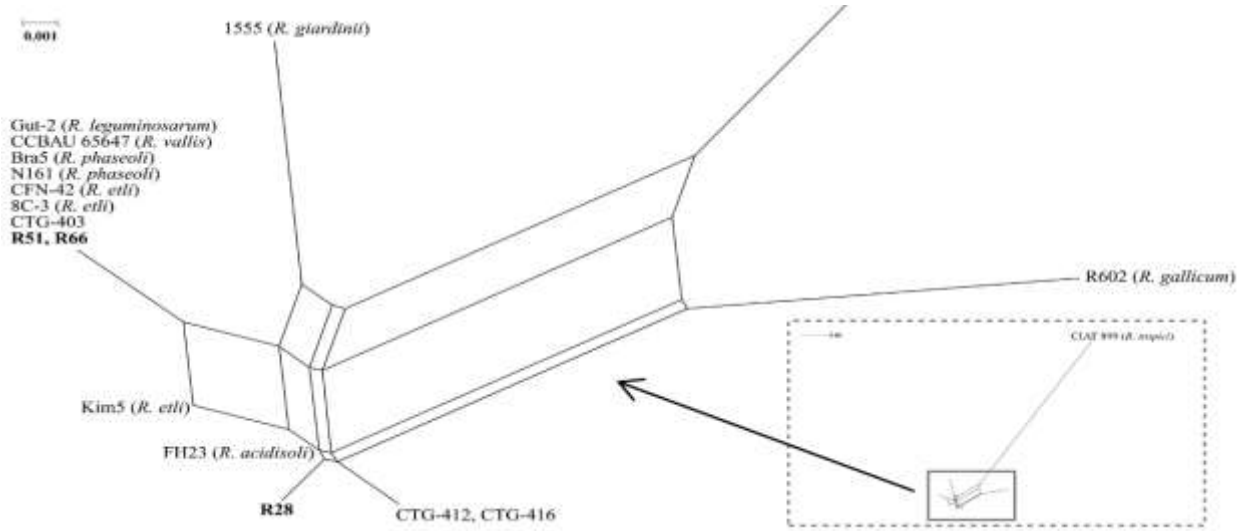

Figure 3. Neighbor network tree derived from *nifH* nucleotide sequences of our isolates, R28 (MH299844), R51 (MH299845), R66 (MH299846) and related *Rhizobium* species obtained from GenBank (Below):
Gut-2_AB740522 (Adhikari et al., Unpublished); CCBAU 65647_GU211767 (Wang et al., 2011); Bra5_CP020898 (Santamaría et al., 2017); N161_CP013587 (Perez-Carrascal et al., Unpublished); CFN-42_U80928 (Girard et al., 1991); 8C-3_CP017243 (Bustos et al., 2017); Kim5_CP021125 (Santamaría et al., 2017); FH23_KJ921065 (Roman-Ponce et al., 2016); R602_AF218126 (Laguerre et al., 2001); CIAT 899_JX863573 (Ricon-Rosales et al., 2013); 1555_KR262797 (Zhao, Unpublished); CTG-403, CTG-412, CTG-416 (Gurkanli et al., 2013)

4. Conclusions and discussion

Rhizobia are classically defined as symbiotic bacteria capable of eliciting and invading root or stem nodules on leguminous plants, where they differentiate into N_2-fixing bacteroids (Tan et al., 2001). Members of this group have been classified within at least 21 genera of *Alphaproteobacteria* and *Betaproteobacteria* (de Lajudie and Young, 2017). *P. vulgaris* L., the host plant we focused on in this study, has known as a promiscuous host which has being nodulated by at least 13 different rhizobial species belonging to the genera *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Bradyrhizobium* and *Burkholderia* (Amarger et al., 1997; Herrera-Cervera et al., 1999; Grange et al., 2004; Han et al., 2005; Martinez-Aguilar et al., 2010). As a result of phylogenetic analyses depeding on the concatenated data set composed of three housekeeping genes, *recA*, *atpD* and *glnII*, our isolate R28 clearly appeared as related to *R. laguerreae* (FB206^T, FB140022, FB310 and FB403) which is not reported as a microsymbiont of *P. vulgaris* L. previously (Figure 1). Phylogenetic analyses of housekeeping genes (especially *recA*, *atpD*, *glnII*, *rpoB*) are very usefull and have been used for description of recent rhizobial species which does not show any differentiation from closely related species in 16S rDNA sequences (Tindall et al., 2010; Mousavi et al., 2015). *R. laguerreae* is a good example for the case that Saidi et al. (2014) showed that there is no 16S rDNA sequence variations between *R. laguerreae*, *R. leguminosarum* and *R. indigoferae*, and that is why we did not sequence the 16S rDNA of our isolates. *R. laguerreae* is identified from rhizobial strains isolated from countries (Spain, Peru and Tunusia) of different continents thus it is obvious that this species has distributed across the World (Saidi et al., 2014). On the other hand, it did not reported from Turkey so far, that is why here we are giving the first report of *R. laguerreae* from Turkey. Another important feature about isolate R28 is, so far all *R. laguerreae* isolates were isolated from *V. faba* L. root nodules thus this isolate is the first *R. laguerreae* sample isolated from a different host (*P. vulgaris* L.) other than faba bean. Our other two isolates R51 and R66 showed closely related *recA*, *atpD* and *glnII* haplotypes with 99.3% nucleotide sequence similarity (possibly indicating the same species) and formed a lineage with *R. sophoriradicis* isolates CCBAU 03470^T and CCBAU 03433. On the otherhand, the nucleotide sequence similarities between isolates R51, R66 and *R. sophoriradicis* isolates were quite low (between 96-96.3%) to consider this lineage as a single species. In our analyses, *recA*, *atpD* and *glnII* nucleotide sequence similarities between sister rhizobial species were between 94% and 98%, the only exception was *R. leguminosarum* and *R. indigoferae* which was 99.4%. From this wiev of point, our isolates are probably representing a new rhizobial species. But this hypothesis needs to be supported with further morphological and molecular analyses. Symbiotic nitrogen fixation genes (*nod*, *nif*, *fix*) of fast growing rhizobia exists on megaplasmids namely symbiotic plasmids (pSym), where exists on symbiotic islands in middle and slow growing rhizobia. These plasmids transfers between different rhizobial species thus phylogenies of the genes carried by pSym do not necessarily corresponds with the chromosomal genes (especially 16S rDNA) and that is why they are useless in taxonomy. Some researchers speculate that phylogeny of nodulation genes may correlate with the host plant because of the convergent evolution thus phylogenetic analyses depending on symbiotic genes gives information for determining the biovars (host range) within rhizobial species (Fischer, 1994; Young and Haukka, 1996; Haukka et al., 1998; Rivas et al., 2009). As the result of network analysis depending on *nodA* sequences (Figure 2), isolate R28 appeared as closely related to PhD12, R51 (and related isolates) and CFN-42 (and related isolates) and showed only 1, 2 and 3 substitutions, respectively (Figure 2). Network of *nifH* partly supported this result (Figure 3). These results indicates that R28 contains a symbiotic plasmid corresponding to biovar. *phaseoli* and that is why we suggest R28 as *R. laguerreae* biovar. *phaseoli*. Our other two isolates, R51 and R66, showed the same *nodA* haplotype with isolates CTG-403 and CTG-416 which we isolated from *P. vulgaris* L. root nodules collected from Black Sea part of Turkey in our previously published study, Gurkanli et al., (2013). This haplotype appeared as related to *nodA* of p42d (*R. etli* isolate CFN-42) and showed only one substitution. Results of *nifH* network also supported *nodA*, that R51 and R66 showed exactly the same *nifH* haplotype with p42d. Depending on the results we can speculate that both of isolates R51 and R66 transferred a symbiotic plasmid of *R. etli* CFN-42 (p42d) from Mexico. This result is not suprising that, it is one of the most common symbiotic plasmid seen in rhizobia nodulating *P. vulgaris* L. in old and new World rhizobial species (Figures 2 and 3).

As conclusion, here we are giving the first report of *R. laguerreae* from Turkey, also reporting this species as microsymbiont of *P. vulgaris* L. for the first time and suggesting R28 as *R. laguerreae* bv. *phaseoli*. Additionally, we are giving the molecular hints for a potentially new rhizobial species close to the *R. sophoriradicis*, but this hypothesis must be supported with further molecular and morphological investigations before giving a latin binomial.

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