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ORIGINAL ARTICLE

Genetic diversity of rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in the Central Black Sea Region of Turkey

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Abstract We have analyzed 30 rhizobial isolates obtained from common bean (*Phaseolus vulgaris* L.) root nodules grown in the Middle Blacksea Region of Turkey, using ARDRA and nucleotide sequence data. ARDRA analysis with enzymes *Cfo*I, *Hinf*I, *Nde*II, *Msp*I and *Pst*I revealed three patterns. Based on sequence data from 16S rDNA, the patterns were identified as, *Rhizobium leguminosarum* bv. *phaseoli* ($n = 16$), *R. etli* bv. *phaseoli* ($n = 8$) and *R. phaseoli* ($n = 6$). On the other hand, nucleotide sequence phylogenies of housekeeping genes (*recA*, *atpD* and *glnII*) selected to confirm the 16S rDNA phylogeny revealed different evolutionary relationships. These results suggested the possibility of lateral transfers of these genes amongst different rhizobial species (including *R. leguminosarum*, *R. etli* and *R. phaseoli*) sharing the same ecological niche (nodulating *P. vulgaris*) which also indicates that there may be no true genetic barier among these species. Phylogenetic analysis based on DNA sequence data from the *nodA* and *nifH* genes showed that all rhizobial species obtained in this study were carrying *nodA* and *nifH* haplotypes which were the same or similar to those of CFN42 (*R. etli* type strain), suggesting a further support for the lateral transfer of CFN42 Sym plasmid, p42, amongst Turkish common bean nodulating rhizobial isolates.

Keywords Rhizobium · Beans · Phylogeny · LGT · Diversity

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Introduction

The genus *Phaseolus* is comprised of nearly 50 species, all distributed in the Americas. Of these, only a few, such as *Phaseolus vulgaris* L. (common bean), *P. coccineus* L. (scarlet runner bean), *P. acutifolius* (A. Gray; tepary bean), *P. polyantus* (Greenm.; polyanthus bean) and *P. lunatus* L. (lima bean) have been domesticated and used as food (Gepts 1990; Martinez-Romero 2003). *P. vulgaris* is the most common *Phaseolus* species with its totally 24.9 million tons (18.3 million tons of dry commonbean and 6.6 million tons of green beans) of production worldwide in 2007 (FAOSTAT). It has been generally accepted that *P. vulgaris* is native to the Americas and some previous studies involving plant morphology, biochemical (phaseolin seed storage protein and isozymes) and molecular (RAPD) analysis have indicated that it was domesticated independently in two separate gene centers with several races; Mesoamerican center (Durango, Mesoamerica, Jalisco, and Guatemala races) and Andean center (Nueva Granada, Peru, Chile), with various ecotypes having been selected for larger seeds and plants (Aguilar et al. 2004; Beebe et al. 2000, 2001; Chacon et al. 2005; Gepts et al. 1986, 1990; Gepts and Bliss 1988; Gepts 1990; Martinez-Romero 2003; Singh et al. 1991a, b, c).

Seeds of the common bean were imported to Europe after the discovery of Americas in 1492 and have been grown there extensively for over 500 years (Gepts and Bliss 1988). The historical records indicate that the cultivation of *P. vulgaris* in Turkey was started much later than in Europe and has a history not longer than 250–300 years (Şehirali 1988).

Phaseolus vulgaris is known to be a relatively permissive host, nodulating effectively with many rhizobial species (Michiels et al. 1998). For many years until 1984, rhizobia nodulating *P. vulgaris* were classified in a single species, *Rhizobium phaseoli*, according to the cross-inoculation concept which was fundamentally based on the ability to

nodulate this plant (Fred et al. 1932). In the first issue of Bergey's *Manuel of Systematic Bacteriology* the bacteria nodulating *P. vulgaris* were grouped into one of the three biological varieties (biovar. *phaseoli*) of *Rhizobium leguminosarum* (Jordan 1984). In the next decade, two new species, *R. etli* bv. *phaseoli* (Segovia et al. 1993) and *R. tropici* (Martinez-Romero et al. 1991), were identified from within North American *R. leguminosarum* bv. *phaseoli* type I and type II isolates, respectively. In the following years, several new species have been identified from the European continent, including *R. giardinii* bv. *phaseoli* and *R. gallicum* bv. *phaseoli* from France (Amarger et al. 1997) and *R. lusitanum* from Portugal (Valverde et al. 2006). Recently, a new species *R. phaseoli* was identified (Ramirez-Bahena et al. 2008). Besides new species, some unexpected rhizobial isolates have also been identified from common bean root nodules such as *Sinorhizobium fredii*-like isolates from Spain (Herrera-Cervera et al. 1999) and *Mesorhizobium* spp. isolates from Brazil (Grange and Hungria 2004). Consequently, nine rhizobial species have so far been reported as microsymbionts of *P. vulgaris*, but it seems that this number will increase in the course of time. Of these species, *R. etli* is the dominant symbiont of both wild and cultivated common bean in Mexico, Colombia and southern Andes and of wild common bean in northwest Argentina, leading to the proposal of coevolution between bean host and *R. etli* since rhizobial species appear to have coevolved with their hosts at their centers of diversification (Mhamdi et al. 2002; Silva et al. 2003; Aguilar et al. 2004). Furthermore, it has been shown that European-originated *R. leguminosarum* bv. *phaseoli* have arisen from the transfer of a pSym from an American-originated *R. etli* bv. *phaseoli* to a *R. leguminosarum* (Segovia et al. 1993). Recently, a similar situation has also been reported for another European-originated rhizobial species, *R. lusitanum*, that harbors genes for symbiosis of both *R. tropici* and *R. etli* (Valverde et al. 2011). These findings may suggest that all other rhizobial species nodulating *P. vulgaris* in Europe, or at least their pSym, might be related to the South-Meso America-originated rhizobial species.

Although common bean is one of the most important crop legumes in Turkey, there have been only a few studies, mostly involving agricultural applications of these bacteria as nitrogen fertilizers, on the rhizobial symbionts of this plant. The study of Küçük et al. (2006) has been the only one dealing with the determination of the rhizobial diversity nodulating *P. vulgaris* in Turkey. The researchers analyzed 30 rhizobial isolates obtained from *P. vulgaris* in Eskişehir (a city in the Aegian part of Turkey) with some phenotypic characters which were insufficient for valid species identification. Thus, our study is the first to characterize, using molecular techniques, rhizobial species nodulating *P. vulgaris* in Turkey.

Our study has two primary objectives. The first is to determine the rhizobial diversity of nodulating *P. vulgaris* in the Middle Black Sea part of Turkey, the second is to analyze the symbiotic genes (*nodA* and *nifH*) of the isolates to characterize the pSym they harbor and follow the possible lateral transfers between the indigenous and exotic (transfered by seeds, etc.) species and isolates.

Materials and methods

Sampling, bacterial isolations and presumption tests

In this study, *P. vulgaris* plants were collected during June–July 2008 from 30 different districts of four cities (Samsun, Sinop, Ordu and Amasya) in the Middle Blacksea part of Turkey (Fig. 1). Bacterial isolation from active root nodules were made according to method of Vincent (1970). Both for isolations and restriking the isolates, standard YMA (Vincent 1970) medium was used. The purity of the isolates were checked by performing some conventional tests, including colony morphology on standard YMA medium (with bromthimol-blue), growth on pepton glucose agar (with bromcresol-purple), and microscopic examination by Gram staining (Vincent 1970; Somasegaran and Hoben 1985; Pollack et al. 2002; Kuykendall et al. 2005).

All candidate rhizobium samples isolated from *P. vulgaris* root nodules were tested for their nodulation ability and symbiotic efficiency on this plant. The nodulation tests were carried out according to the method of Vincent (1970) with three replicates for each isolate. After 4 weeks of incubation in a growth chamber at 14 h light and 10 h dark, isolates were evaluated for their nodulation ability and symbiotic efficiency.

DNA isolation and PCR amplifications

For genomic DNA extractions, bacteria were grown in TY (Tryptone Yeast Extract) broth media (Ditta et al. 1987) for 2 days in a rotary incubator fixed at 225 rpm and 28 °C. Because the isolates produced too much exopolisaccarides, genomic DNA extractions were made with the CTAB/NaCl miniprep method and were stored in −20 °C until used (Temizkan and Arda 2004).

The PCR protocols and DNA primers shown in Table 1 were used to amplify 16S rDNA, *recA*, *atpD*, *glnII*, *nodA* and *nifH* gene fragments in a MWG Primus thermal cycler. For all genes, amplifications were performed in a 50 µl volume containing template DNA<0.5 µg, 1.5 mM $MgCl_2$ (1.75 mM $MgCl_2$ was used for the amplification with primers nifH1/nifH2), 1.25 U Taq polymerase (Promega, Go-Taq Flexi DNA Polymerase), 0.8 mM dNTP mix (Amresco, Solon, OH, USA), 10 µl of 5X PCR buffer (Promega, Go-Taq Green Buffer), 0.4 pmol of each primer in final concentration (0.6 pmol used for primers nifHF/nifHI) and ddH_2O. The PCR

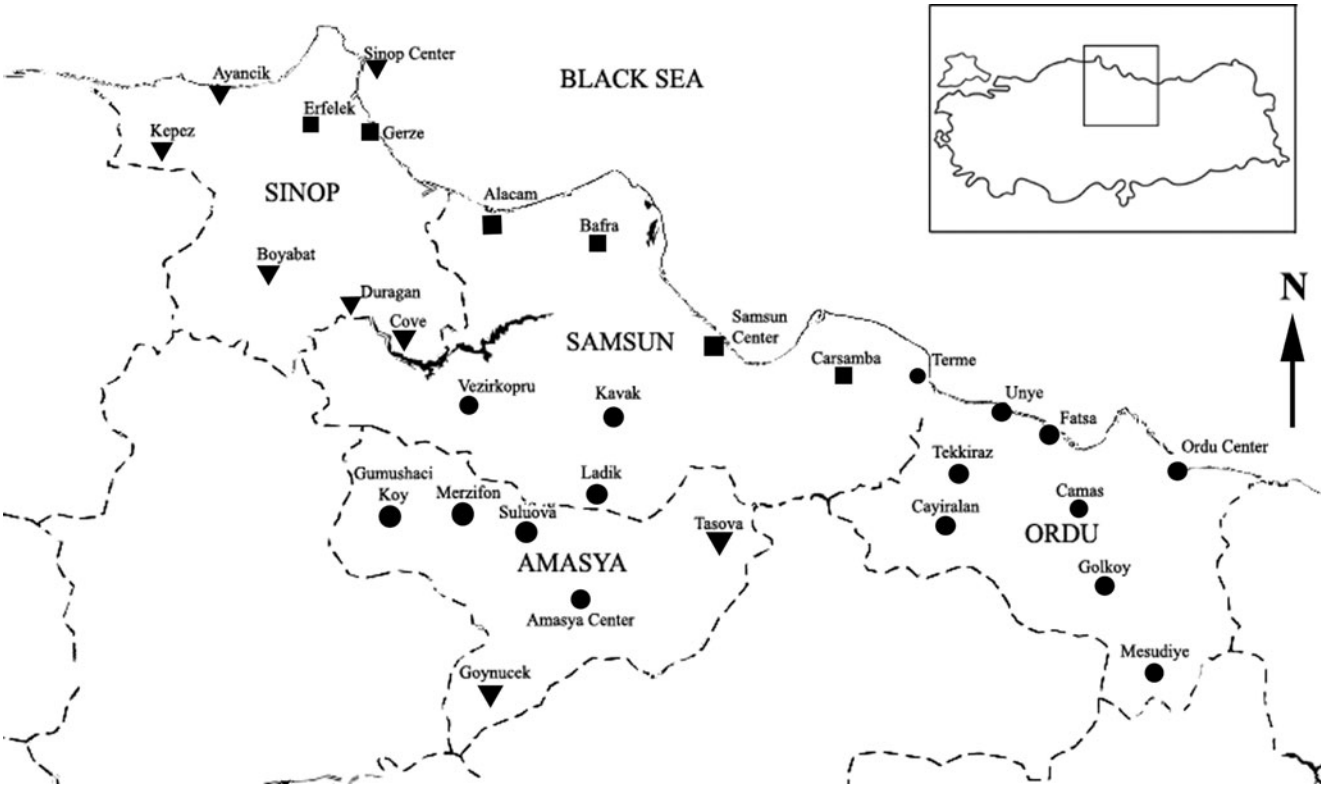

Fig. 1 The localities where *Phaseolus vulgaris* L. plant samples were collected. *Triangle* shape is indicating the localities which taxonomically uncertain samples were isolated, *Square* shape is indicating the localities which *R. phaseoli*-related samples were isolated, *Circle* shape is indicating the localities which *R. leguminosarum bv. phaseoli* samples were isolated.

products were electrophoresed on 1 % agarose gel (Amresco) prepared in 1X TBE (Tris-Borate-EDTA) buffer. After staining with ethidium bromide, gels were visualized with the GeneGenius Bio imaging system (Syngene; Synoptics Group, Cambridge, UK).

When the degenerate primers (primers for *nodA* and *nifH* genes) produced multiple PCR products, the appropriate sized DNA band was cut from the gel and kept in a microcentrifuge tube containing 50 μl ddH_2O for 1 h. Then, 10 μl of this stock was used as template for the second PCR reaction (Haukka et al. 1998).

Purification and PCR-RFLP analysis of 16S rDNA gene

The 16S rDNA PCR products (amplified with fD1/rD1) were purified to be used for RFLP analysis using a

Table 1 PCR protocols and primers used in this study

Gene	Primer	ID	C	D	A	E	FE
16S rDNA	fD1/rD1 (Weisburg et al. 1991)	95 °C / 5 min	×35	95 °C / 45 s	55 °C / 45 s	72 °C / 2 min	72 °C / 4 min
16S rDNA	pA/pF (Zhang et al 1999)	95 °C / 3 min	×35	95 °C / 1 min	55 °C / 1 min	72 °C / 1 min	72 °C / 5 min
recA	recA-Forward/recA-Reverse (Gaunt et al. 2001)	95 °C / 5 min	×30	95 °C / 45 s	50 °C / 1 min	72 °C / 1 min	72 °C / 2 min
nodA	nodA1/nodA2 (Haukka et al. 1998)	95 °C / 5 min	×35	95 °C / 45 s	49 °C / 45 s	72 °C / 45 s	72 °C / 5 min
nodA	nodA1/nodA3 (Zhang et al. 2000)	97 °C / 2 min	×35	92 °C / 40 s	56 °C / 1 min	72 °C / 90 s	72 °C / 5 min
atpD	atpD273F/atpD771R (Gaunt et al. 2001)	95 °C / 3 min	×35	94 °C / 1 min	52 °C / 1 min	72 °C / 1 min	72 °C / 5 min
glnII	glnII12F/glnII689R (Vinuesa et al. 2005a)	95 °C / 3 min	×35	94 °C / 1 min	55 °C / 1 min	72 °C / 1 min	72 °C / 5 min
nifH	nifH1/nifH2 (Eardly et al. 1992)	95 °C / 5 min	×40	95 °C / 45 s	53 °C / 1 min	72 °C / 1 min	72 °C / 5 min
NifH	nifHI/nifHF (Laguerre et al. 2001)	95 °C / 5 min	×40	94 °C / 1 min	59 °C / 1 min	72 °C / 1 min	72 °C / 5 min

ID Initial denaturation, *C* PCR cycle number, *D* denaturation, *A* annealing, *E* extension, *FE* final extension

QIAquick PCR Purification Kit as specified by the manufacturer. The PCR-RFLP analysis of 16S rDNA gene was performed with the restriction enzymes, *Hinf*I (New England BioLabs), *Msp*I (Fermentas), *Nde*II (Promega) and *Cfo*I (Promega) (Laguerre et al. 1994). Additionally, the samples that showed an RFLP pattern similar to *R. phaseoli* with these four enzymes were also digested with *Pst*I (Sigma). All restriction reactions were prepared in 10 μl volume as specified by the manufacturer. The digested bands were separated on 2.5 % metaphore agarose gel (Lonza, USA) prepared in 1× TBE buffer.

16S rDNA, *recA*, *atpD*, *glnII*, *nodA* and *nifH* Gene sequencings and phylogenetic analysis

The 16S rDNA, *recA*, *atpD*, *glnII*, *nodA* and *nifH* nucleotide sequencings of the selected rhizobial isolates were performed in both directions with the same primers used for the PCR amplifications (Table 1). For 16S rDNA nucleotide sequencing, we used an internal primer set pA/pF (Zhang et al. 1999) addition to full length reading with primers fD1/rD1 (Weisburg et al. 1991) for a more reliable sequencing. The nucleotide sequencings were made commercially by Macrogen, Korea, using the sequencer Roche 454 GS-FLX Titanium. Sequences were checked and edited using the program SeqMan II module of the LASERGENE 99 system (Applied Biosystems). Multiple nucleotide sequence alignments were generated using ClustalX (Thompson et al. 1997) and optimized by hand using BioEdit (Hall 1999). To determine the most appropriate DNA substitution model for our datasets, the Akaike information criterion (AIC) (Akaike 1974) and Bayesian information criterion (BIC) tests were applied with jModelTest v. 0.1 package program (Guindon and Gascuel 2003; Posada 2008). To evaluate the phylogenetic relationships among isolates, Neighbor-Joining (NJ) (Saitou and Nei 1987) and Maximum-Parsimony (MP) (Eck and Dayhoff 1966; Fitch 1977) methods were used. Both analysis were made using PAUP* v.4.0b10 (Swofford 1998). The heuristic search approach was applied for the MP analyses using TBR swapping algorithm with 10 random repetitions. Then, strict and 50 % majority rule consensus trees were generated from equally parsimonious trees. To test the robustness of the phylogenetic trees, bootstrap tests (Efron 1982; Felsenstein 1985) was applied. For NJ trees, bootstrap analysis was conducted with 10,000 pseudo-replicates using the same substitution models selected for the phylogenies, and for MP trees, bootstrap anaysis was conducted with 1,000 pseudo-replicates with 10 random repetitions for each replication.

For the isolates showing significant topological differences in their trees derived from 16S rDNA, *recA atpD* and *glnII* genes, the ILD (Incongruence Length Difference) test (Farris et al. 1994) was performed to see any possible horizontal gene transfer among different isolates and species. The ILD test was performed using PAUP* v.4.0b10 with 1,000 random partition replicates.

Accession numbers for our 16S rDNA, *recA*, *atpD*, *glnII*, *nodA* and *nifH* sequences are given in Table 2.

Results

Isolates and conventional tests

A total of 30 rhizobial isolates (Table 2) were collected from common bean grown in four different provinces (Samsun, Ordu, Sinop and Amasya) in the Middle Blacksea region of Turkey (Fig. 1). All were rod-shaped and Gram-negative cells, and none of them grew on PGA or changed the pH of this media, suggesting that all isolates had the typical characteristics of a rhizobium and contained no contamination. After 4 weeks of incubation, the isolates produced active (pink-colored) determinate nodules on *P. vulgaris* roots, indicating that they harbored proper pSym for symbiosis with this host plant.

PCR-RFLP analysis of the 16S rDNA gene

Nearly full length 16S rDNA gene were amplified for all 30 rhizobium isolates with primers fD1/rD1 and digested with restriction enzymes, *Hinf*I, *Cfo*I, *Msp*I and *Nde*II. Additionally, we theoretically digested the 16S rDNA sequences of the rhizobial type strains (obtained from GenBank) with BioEdit (Hall 1999) for comparasion. Fourteen of our isolates showed a RFLP pattern (ABAB) similar to *R. phaseoli* type strain ATCC 14482, and 16 of them showed a pattern (AAAA) different from any known rhizobial species (Fig. 2; Table 2). However, our further analyses (sequence phylogenies) indicated that some of our isolates with *R. phaseoli* RFLP pattern were phylogenetically related to *R. etli*. This was a suprising result because *Cfo*I seemed to be discriminative for these two species when we theoretically digested the type strains of *R. phaseoli* (ATCC 14482) and *R. etli* (CFN42). When we aligned our 16S rDNA sequences with the type strains, we determined a transversion (C→G) substitution at the 323th nucleotide position of our *R. etli*-related sequences. This mutation generates an extra digestion site for *Cfo*I just like in *R. phaseoli*. This substitution seems to be common, because an American rhizobial isolate, USDA 2667, also has the same substitution at the same position. To discriminate between the two species, we also digested our 14 samples with *R. phaseoli* RFLP pattern using *Pst*I enzyme (determined with BioEdit), resulting in *R. phaseoli* (ABABA) pattern for 6 isolates and *R. etli* (ABABB)-like patterns for 8 isolates (Fig. 2; Table 2). 16S and *recA* DNA phylogenies of representetive isolates of

Table 2 16S rDNA PCR-RFLP patterns and accession numbers for 16S rDNA, *recA*, *atpD*, *glnII*, *nodA* and *nifH* genes of the *Rhizobium* isolates obtained in this study

Isolate	Geographical origin	16S rDNA-RFLP Pattern					DNA Sequence accession number					
		CfoI	*HinfI*	*MspI*	*NdeII*	*PstI*	16S rDNA	*recA*	*atpD*	*glnII*	*nodA*	*nifH*
CTG-401	ORDU-Centre	A	A	A	A	–						
CTG-402	ORDU-Fatsa	A	A	A	A	–						
CTG-403	ORDU-Unye	A	A	A	A	–	JX971653	JX971661	JX971669	JX971677	JX971685	JX971693
CTG-404	ORDU-Tekkiraz	A	A	A	A	–						
CTG-405	ORDU-Cayiralan	A	A	A	A	–						
CTG-406	ORDU-Camas	A	A	A	A	–						
CTG-407	ORDU-Golkoy	A	A	A	A	–	JX971654	JX971662	JX971670	JX971678	JX971686	JX971694
CTG-408	ORDU-Mesudiye	A	A	A	A	–						
CTG-413	SAMSUN-Terme	A	A	A	A	–						
CTG-414	SAMSUN-Kavak	A	A	A	A	–						
CTG-415	SAMSUN-Ladik	A	A	A	A	–						
CTG-416	SAMSUN-Vezirkopru	A	A	A	A	–	JX971655	JX971663	JX971671	JX971679	JX971687	JX971695
CTG-425	AMASYA-Center	A	A	A	A	–						
CTG-426	AMASYA-Suluova	A	A	A	A	–						
CTG-427	AMASYA-Merzifon	A	A	A	A	–	JX971656	JX971664	JX971672	JX971680	JX971688	JX971696
CTG-428	AMASYA-G.Hacikoy	A	A	A	A	–						
CTG-409	SAMSUN-Center	A	B	A	B	A						
CTG-410	SAMSUN-Bafra	A	B	A	B	A						
CTG-411	SAMSUN-Alacam	A	B	A	B	A						
CTG-412	SAMSUN-Carsamba	A	B	A	B	A	JX971657	JX971665	JX971673	JX971681	JX971689	JX971697
CTG-418	SİNOP-Erfelek	A	B	A	B	A						
CTG-419	SİNOP-Gerze	A	B	A	B	A	JX971658	JX971666	JX971674	JX971682	JX971690	JX971698
CTG-417	SİNOP-Center	A	B	A	B	B						
CTG-420	SİNOP-Ayancik	A	B	A	B	B						
CTG-421	SİNOP-Kepez	A	B	A	B	B						
CTG-422	SİNOP-Boyabat	A	B	A	B	B						
CTG-423	SİNOP-Duragan	A	B	A	B	B	JX971659	JX971667	JX971675	JX971683	JX971691	JX971699
CTG-424	SİNOP-Cove	A	B	A	B	B						
CTG-429	AMASYA-Tasova	A	B	A	B	B						
CTG-430	AMASYA-Goynucek	A	B	A	B	B	JX971660	JX971668	JX971676	JX971684	JX971692	JX971700

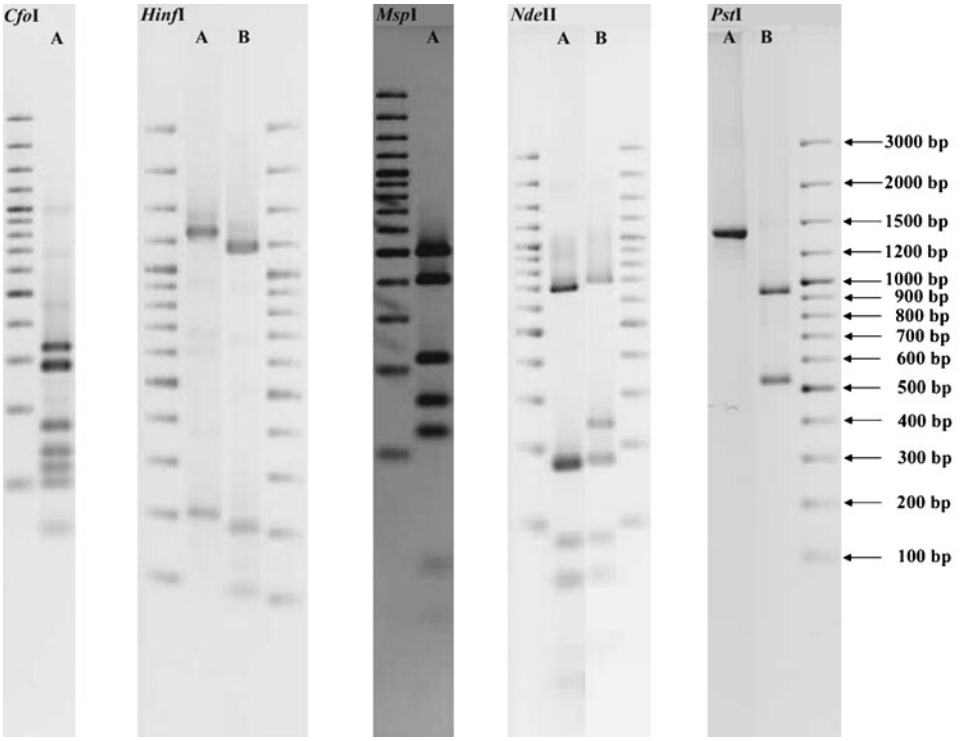

Fig. 2 16S rDNA PCR-RFLP patterns derived from digestions with restriction enzymes *Cfo*I, *Hinf*I, *Msp*I, *Nde*II and *Pst*I. GeneRuler, 100-bp Plus DNA Ladder (Fermentas) was used to estimate the sizes of digested nucleotide bands

AAAA RFLP pattern not matching with any known rhizobial species suggested their close relationships with *R. leguminosarum* type strain (USDA 2370). The incongruence in between the RFLP patterns of our *R. leguminosarum* bv. *phaseoli* (Rlp) isolates and that of the USDA 2370 was due to *Cfo*I digestion resulting from a transvertion (C→G) mutation at the 321th base of our isolates. This led us to choose a total of eight isolates representing the different RFLP patterns and different geographical location of the studied provinces for 16S rDNA, *recA*, *atpD*, *glnII*, *nodA* and *nifH* nucleotide sequencings (Table 2).

DNA sequencings and phylogenetic analysis

We sequenced approximately 1,350 bp of the 16S rDNA gene in eight representative isolates. Phylogenetic analysis of our isolates together with the rhizobial strains obtained from GenBank (see the legend of Fig. 3a, b) were carried out with 1,275 aligned nucleotides which contain 124 variable sites. The NJ (Fig. 3a) tree was generated using TIM2+I (I: 0.865) substitution model which showed the highest bootstrap values. In the 16S rDNA NJ tree, our isolates were clustered in three monophyletic groups. CTG-412 and CTG-419, chosen as representatives for the isolates showing the ABABA 16S rDNA RFLP pattern, were grouped with *R. phaseoli* type strain (ATCC 14482) and showed 99.9 % nucleotide similarity with it (Table 3). In the second group, representative isolates, CTG-423 and CTG-430, for the ABABB 16S rDNA RFLP pattern were found to be related to *R. etli* type strain CFN42 (99.7 % nucleotide similarity) and with another *R. etli* isolate, USDA 2667 (99.9 % nucleotide similarity). Three (CTG-407, -416, -427) of four representative isolates chosen for the AAAA 16S rDNA RFLP pattern showed the same 16S rDNA haplotype with *R. leguminosarum* bv. *trifolii* strain ATCC 14480 (and also with with ARPV02 from Argentina and CCBAU 43229 from China), and they also showed 99.6 % DNA similarity with *R. leguminosarum* type strain USDA 2370 and *R. leguminosarum* bv. *phaseoli* isolate USDA 2671 (Table 3). The fourth isolate, CTG-403, was also gathered within the same monophyletic group with USDA 2370 and other *R. leguminosarum* isolates ATCC 14480 and USDA 2671, with 99.6, 99.8, and 99.6 % DNA similarities, respectively (Fig. 3a). In the MP analyses, we determined six most parsimonious trees (length: 199; CI: 0.749; RI: 0.860). Although the MP tree showed the same main monophyletic groups with the NJ tree, there were some topological differences in the relationships among these main groups (Fig. 3b). But, in general, our isolates were found to be related to the same species both in the NJ and MP trees (Fig. 3a–b).

To confirm the 16S rDNA-based identifications of our isolates, we also partially sequenced (approx. 550 bp) their *recA* genes and inferred their phylogenetic relationships over 414 bases (132 variable sites) aligned with the *recA* haplotypes dowloaded from GenBank (see the legend of Fig. 4a, b). The AIC and BIC tests suggested GTR+I (I: 0.650) and TIM2+I (I: 0.651) substitution models, respectively. NJ trees produced with these models showed similar bootstrap values for the same nodes, but the tree with the TIM2+I substitution model seemed to be more reliable in

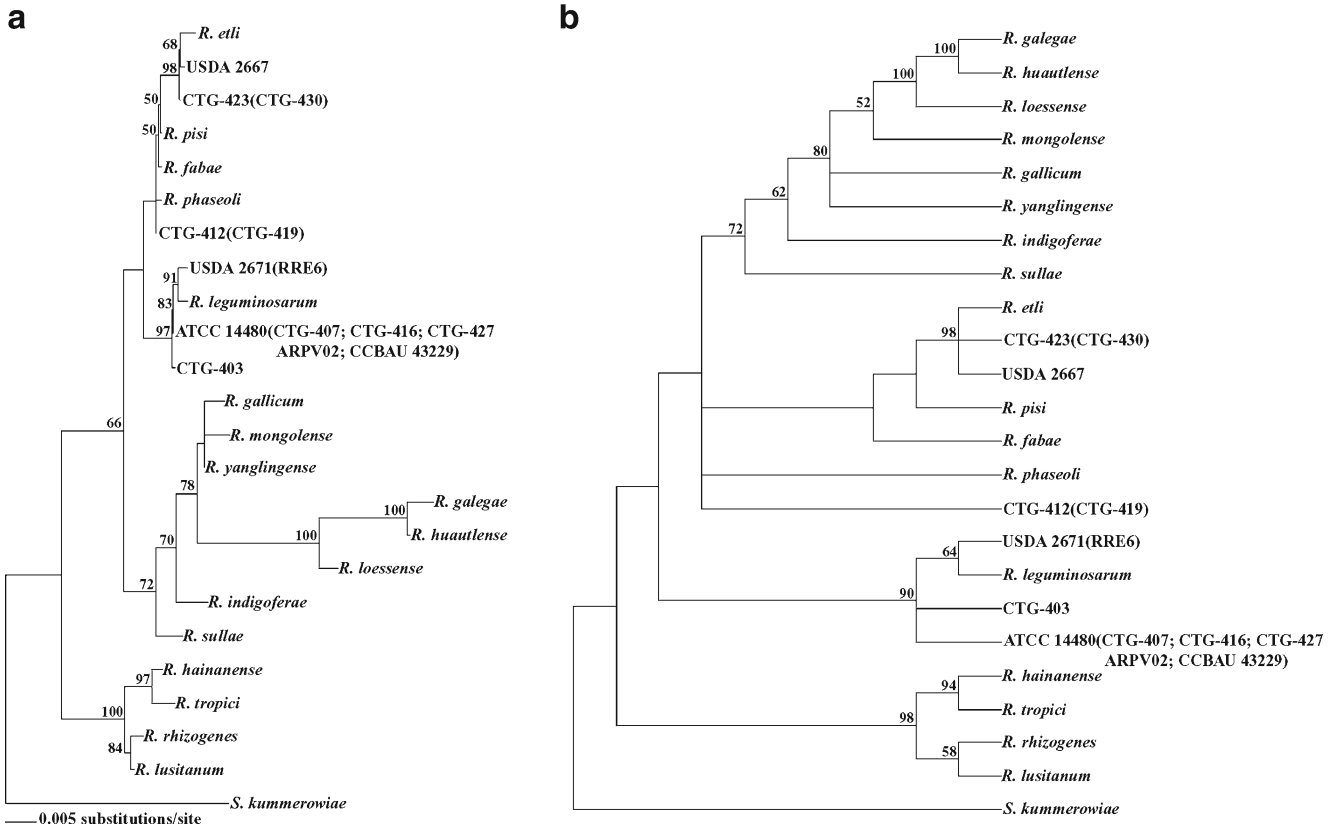

Fig. 3 NJ (**a**) and MP (**b**) trees showing the phylogenetic relationships of 16S rDNA haplotypes obtained in this study and the ones obtained from GenBank (stated below). On the trees, only the bootstrap values greater than 50 % are shown. *R. leguminosarum*^{T} (USDA 2370) U29386; *R. etli*^{T} (CFN42) U28916; USDA 2671 U29388 (Van Berkum et al. 1996); *R. fabae*^{T} (CCBAU 33202) DQ835306; CCBAU 43229 DQ835308 (Tian et al. 2007); *R. hainanense*^{T} (CCBAU 57015) U71078 (Chen et al. 1997); *R. gallicum*^{T} (R6002sp) U86343 (Amarger et al. 1997); *R. mongolense*^{T} (USDA 1844) U89817 (Van Berkum et al. 1998); *R. yanglingense*^{T} (SH22623) AF003375 (Tan et al. 2001); *S. kummerowiae*^{T} AY034028; *R. indigoferae*^{T} (CCBAU 71042) AF364068 (Wei et al. 2002); *R. sullae*^{T} (IS123) Y10170 (Squartini et al. 2002); *R. rhizogenes*^{T} (IFO 13257) D01257 (Sawada et al. 1993); *R. lusitanum*^{T} (P1-7) AY738130 (Valverde et al. 2006); *R. tropici*^{T} (CIAT 899) U89832 (Van Berkum et al. 1998); *R. pisi*^{T} (DSM 30132) AY509899; *R. phaseoli*^{T} (ATCC 14482) EF141340 ATCC 14480 AY509900 (Ramirez-Bahena et al. 2008); *R. galegae*^{T} (LMG 6214) X67226 (Willems and Collins 1993); *R. huautlense*^{T} (SO2) NR_024863 (Wang et al. 1998); *R. loessense*^{T} (CCBAU 7190B) AY034029 (Wei et al. 2003); ARPV02 AY196964 (Abril et al. 2007); USDA 2667 U47303 (Buttery et al. 1997); RRE6 AY946012 (Singh et al. 2006); Mim-2 DQ648574 (Chen, unpublished)

the meaning of main monophyletic groups when we compare it with the available literature. And the tree drawn with this model also gave bootstrap values for more internal nodes than the one with the GTR+I substitution model. Thus, the *recA* NJ tree generated using TIM2+I substitution model was discussed (Fig. 4a). In the tree, isolates CTG-403 and CTG-427, which were found to be related to *R. leguminosarum* in the 16S rDNA trees, were also grouped within this lineage that contains *R. leguminosarum* type strain (USDA 2370) and *R. leguminosarum* bv. *trifolii* isolate (ATCC 14480) in *recA* phylogeny. The nucleotide sequence identity among this lineage was between 98 and 99.5 %. On the other hand, our other two *R. leguminosarum* related isolates, CTG-407 and CTG-416, plus isolate CCBAU 43229 (from *V. faba* in China), showed a totally different *recA* haplotype from any known rhizobial species and formed a separate monophyletic group far apart from *R. leguminosarum* (Fig. 4a). Isolate CTG-407 showed 93.7, 92.5, 92.9, and 94.9 % nucleotide similarities with the type strains of *R. leguminosarum*, *R. fabae*, *R. pisi*, and isolate CTG-416, respectively, where isolate CTG-416 showed 94.4, 93.9, and 93.7 % nucleotide similarities with the same type strains. Isolates CTG-423 and CTG-430 identified as *R. phaseoli*-related were also found to be related to this species in *recA* phylogeny with a 95.1 % nucleotide similarity (Table 3). For isolates CTG-412 and CTG-419, we determined an incongruence between 16S rDNA and *recA* phylogenies similar to the one just mentioned. These isolates were grouped with *R. etli* type strain CFN42 and with other *R. etli* isolates in 16S rDNA NJ and MP trees (Fig. 3a, b), whereas they were grouped with *R. phaseoli* type strain ATCC 14482 and *R. phaseoli* isolates obtained in this study in the *recA* tree. The nucleotide similarity between the *R. phaseoli* type strain and

Table 3 Table showing the 16S rDNA, *recA*, *atpD* and *glnII* nucleotide sequence similarities among rhizobial isolates obtained in this study and the type strains of *R. leguminosarum* (USDA 2370), *R. etli* (CFN42) and *R. phaseoli* (ATCC 14482) species respectively

	16S rDNA	*recA*	*atpD*	*glnII*
CTG-403	99.6 %; 98.8 %	98 %; 90.8 %	92.4 %; 90.7 %	97.3 %; 92.1 %
	99.2 %	91 %	92.9 %	90.3 %
CTG-407	99.6 %; 98.8 %	93.7 %; 90.8 %	93.6 %; 90.2 %	92.7 %; 94.1 %
	99.2 %	91 %	93.1 %	91.7 %
CTG-416	99.6 %; 98.8 %	94.4 %; 91.3 %	92.4 %; 91.2 %	97.7 %; 92.1 %
	99.2 %	91.5 %	92.9 %	90.3 %
CTG-427	99.6 %; 98.8 %	100 %;90.5 %	99.5 %; 89.5 %	99.5 %; 91.5 %
	99.2 %	90.8 %	91.4 %	90.1 %
CTG-412	99.1 %; 99.4 %	92.7 %; 91 %	94.8 %; 90.4 %	92.9 %; 95.1 %
	99.9 %	95.6 %	92.1 %	93.7 %
CTG-419	99.1 %; 99.4 %	92.5 %; 90.8 %	94.6 %; 90.2 %	92.9 %; 95.1 %
	99.9 %	95.8 %	91.9 %	93.7 %
CTG-423	98.7 %; 99.7 %	92 %; 91 %	91.9 %; 90.7 %	92.3 %; 94.7 %
	99.5 %	95.1 %	92.1 %	93.5 %
CTG-430	98.7 %; 99.7 %	92 %; 91 %	91.9 %; 90.7 %	92.3 %; 94.7 %
	99.5 %	95.1 %	92.1 %	93.5 %

isolates CTG-412 and CTG-419 were 95.6 and 95.8 %, respectively; on the other hand, *R. etli* type strain showed only 91 and 90.8 % nucleotide similarities (Table 3). These groupings seem to be robust, having relatively high bootstraps. MP analyses resulted in three most parsimonious trees (length: 311 step; CI: 0.566; RI: 0.618). All the similar groupings and incongruences mentioned for the NJ tree were also determined in the MP tree (Fig. 4b).

Another housekeeping gene we analyzed to determine the phylogenetic relationships was *atpD* which codes for the ATP synthase beta subunit. Using the primers mentioned in Table 1, we sequenced approximately 520 bp of the gene and inferred their phylogenies over 410 nucleotides (with 114 variable sites) aligned with the *atpD* haplotypes dowloaded from GenBank (see the legend of Fig. 5a, b). Both AIC and BIC tests suggested TIM2+I+G (I: 0.558; G: 0.944) substitution model. In the NJ tree (Fig. 5a), only the isolate CTG-427 grouped within the typical *R. leguminosarum* clade comprised of USDA 2370 (*R. leguminosarum* type strain) and ATCC 14480 (*R. leguminosarum* bv. *trifolii* type strain) with 99.5 and 98.2 % nucleotide similarities, respectively. On the other hand, other potential *R. leguminosarum* isolates (due to 16S rDNA and *recA* phylogenies), CTG-403, CTG-407, and CTG-416 placed in between the *R. leguminosarum* clade and the one comprised of *R. fabae* and *R. pisi* type strains. These isolates showed 92.4, 93.6, and 92.4 % nucleotide similarities with *R. leguminosarum* type strain where it showed approx. 93.1, 93.1, and 92.8 % average nucleotide similarities with the *R. fabae* and *R. pisi* clade. Interestingly, our isolates CTG-412 and CTG-419 that showed 16S rDNA and *recA* haplotypes similar to *R. phaseoli* were found to be a close sister to the *R. leguminosarum* clade with a 81 % bootstrap value. The nucleotide similarities of CTG-412 and CTG-419 with USDA 2370 were 94.8 and 94.6 %, respectively, where was 92.1 and 91.9 % with *R. phaseoli* (Table 3). Likewise, isolates CTG-423 and CTG-430 that showed 16S rDNA and *recA* haplotypes similar to *R.etli* and *R. phaseoli*, respectively, was placed between the *R. leguminosarum* clade and the *R. fabae–R. pisi* clade and showed 91.9, 93.4, 93.9, 92.1, and 90.7 % nucleotide similarities with *R. leguminosarum*, *R. fabae*, *R. pisi*, *R. phaseoli*, and *R. etli* type strains, respectively. MP analyses gave 11 most parsimonious trees (length: 277 step; CI: 0.542; RI: 0.666). The consensus MP tree showed the same topology with the NJ tree (Fig. 5b).

In addition to 16S rDNA, *recA* and *atpD* we partially sequenced (approx. 640 bp) *glnII* gene of our isolates and inferred their phylogenetic relationships over 497 bases (141 variable sites) aligned with the *glnII* haplotypes dowloaded from GenBank (see the legend of Fig. 6a–b). The AIC and BIC tests suggested TIM1+I+G (I: 0.499; G: 0.748) and TIM1+G (G: 0.197) substitution models, respectively. Because it has higher bootstrap values, the NJ tree created with TIM1+I+G substitution model has prefered to give in this study (Fig. 6a). In the NJ tree, isolates CTG-403, CTG-416 and CTG-427 were appeared in *R. leguminosarum* lineage and showed 97.3 %, 97.7 % and 99.5 % nucleotide similarity respectively with USDA 2370 (Table 3). This group seemed to be very robust due to the high bootstrap values. Surprisingly isolate CTG-407, that mostly appeared as close to *R. leguminosarum* lineage grouped with *R. etli* (CFN42) with a 94.1 % nucleotide similarity (Fig. 6). Coherent with the *recA* phylogeny, isolates CTG-412 (CTG419) and CTG-423 (CTG-430) placed sister to *R. phaseoli* type strain (ATCC 14482) with 93.7 and 93.5 % nucleotide similarities, respectively (Table 3). MP analyses gave two most parsimonious trees (length: 296 step; CI:

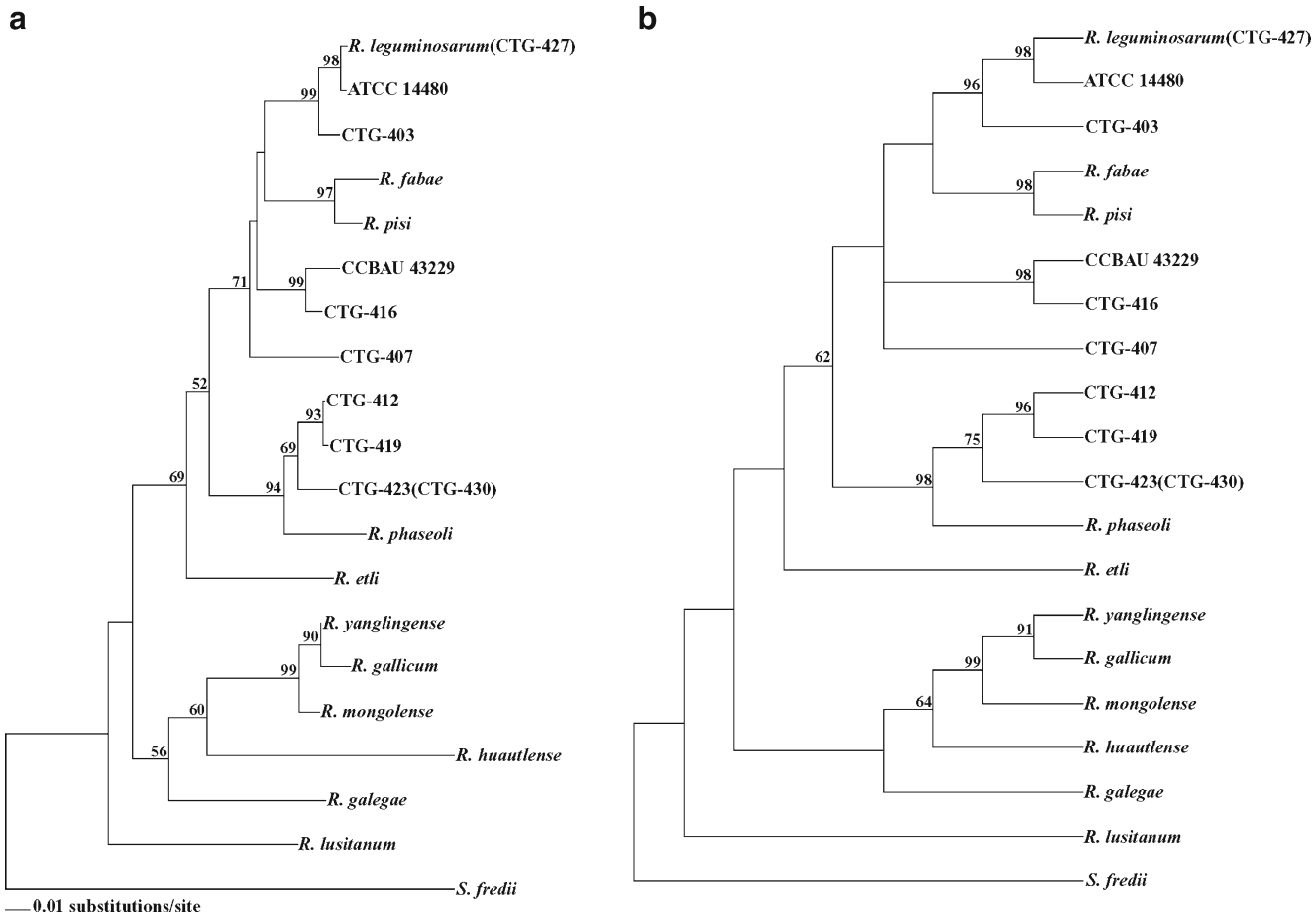

Fig. 4 NJ (**a**) and MP (**b**) trees showing the phylogenetic relationships of *recA* haplotypes obtained in this study and the ones obtained from GenBank (stated below). On the trees, only the bootstrap values greater than 50 % are shown. *R. leguminosarum*^{T} (USDA 2370) AJ294376; *R. galegae*^{T} (USDA 4128) AJ294378; *R. etli*^{T} (CFN42) AJ294375 (Gaunt et al. 2001); ATCC 14480 EF113135 (Santillana et al. 2008); *R. fabae*^{T} (CCBAU 33202) EF579941 (Tian et al. 2008); *R. phaseoli*^{T} (ATCC 14482) EF113136; *R. pisi*^{T} (DSM 30132) EF113134 (Ramirez-Bahena et al. 2008); CCBAU 43229 GQ323665 (Tian et al. 2010); *R. gallicum*^{T} (R6002sp) AY907357; *R. mongolense*^{T} (USDA 1844) AY907358; *R. yanglingense*^{T} (SH22623) AY907359; *R. huautlense*^{T} (SO2) AY688601 (Vinuesa et al. 2005b); *R. lusitanum*^{T} (p1-7) DQ431674 (Valverde et al. 2006), *S. fredii*^{T} (USDA 205) AJ294379 (Gaunt et al. 2001)

0.611; RI: 0.692). The consensus MP tree (Fig. 6b) supported the lineages and the relationships we had in the NJ tree.

To investigate the incongruences between the genes analyzed and to provide insights into the lateral transfer possibilities of these genes between rhizobial isolates and species, we performed an ILD test for a set of organisms including our isolates and the Chinese CCBAU 43229 in addition to *R. leguminosarum*, *R. trifolii*, *R. fabae*, *R. pisi*, *R. etli*, and *R. phaseoli* type strains. We performed the ILD test for every binary combination of the genes analyzed in this study. As a result, all tests gave the *P* value of 0.001 [*P*=1−(999/1,000)], suggesting only one tree derived from 1,000 random partition replicates gave a length smaller or equal to the length of the original partition tree, suggesting possible lateral transfers between rhizobial isolates and species.

For *nodA* amplifications, we initially used the nodA1/nodA2 primer set as explained in Haukka et al. (1998), but we could not amplify a suitably sized band (660 bp). Thus, we tried nodA1/nodA3 primer set (Zhang et al. 2000) and amplified approx. 600 bp of *nodA* gene and sequenced the fragment with the same primers. The phylogenetic relations of the *nodA* haplotypes found in this study and those from GenBank (see the legend of Fig. 7a–b) were conducted using 484 aligned nucleotides with 392 variable sites. AIC and BIC analysis suggested TVM+G (G: 0.818) and HKY+G (G: 0.815) substitution models, respectively. The NJ tree was generated with the TVM+G model (Fig. 7a) since it gave higher bootstrap values. All our isolates except for CTG-412 had the same *nodA* haplotype although they were from three different rhizobial species. They formed a monophyletic group with CFN42 (and also with Ro84 and CIAT 652), *R. gallicum* type strain and ICMP 2672 (*R. leguminosarum* bv. *phaseoli* isolate) and showed 99.7, 99.3, and 99.5 % nucleotide similarities with these isolates, respectively.

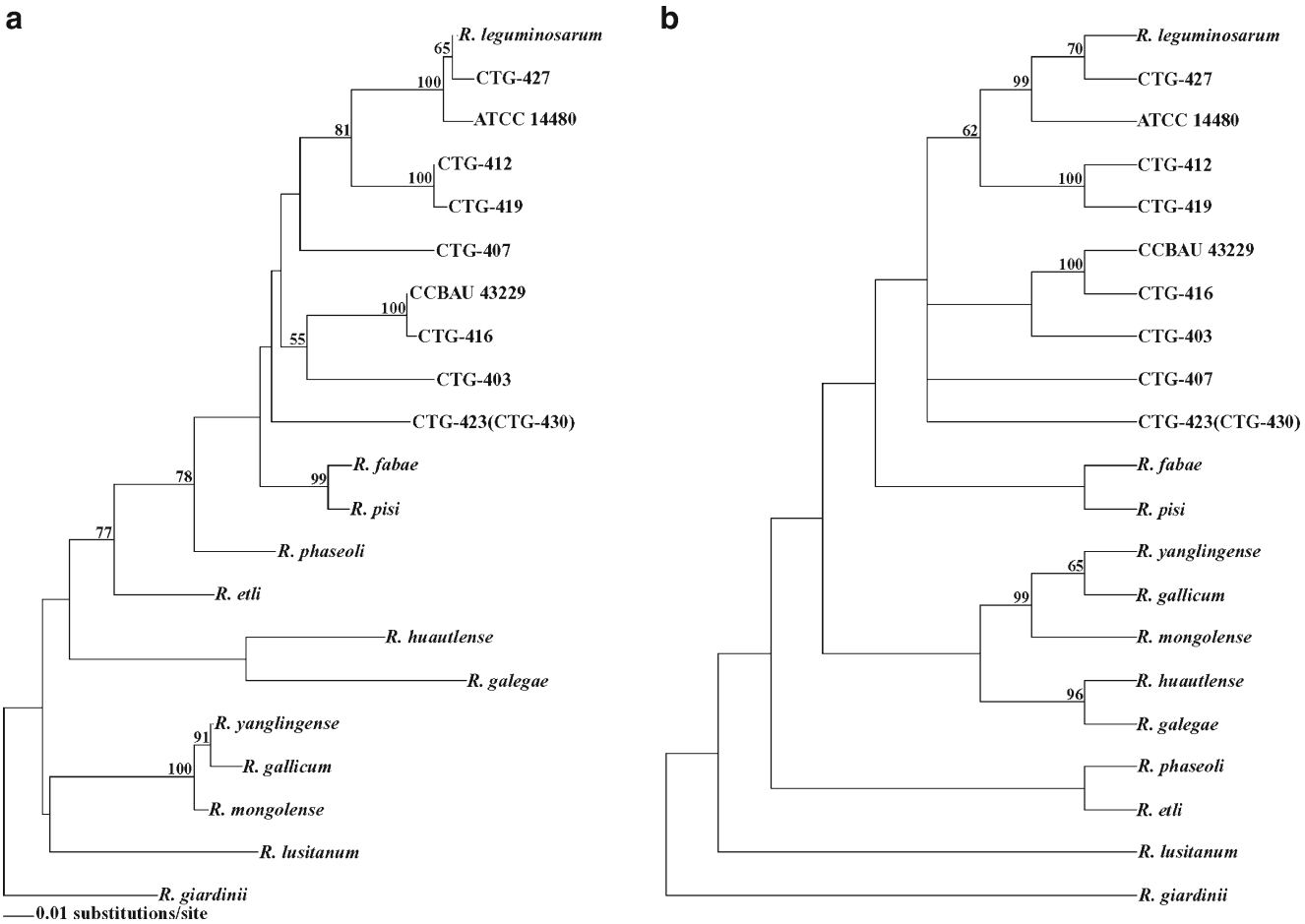

Fig. 5 NJ (**a**) and MP (**b**) trees showing the phylogenetic relationships of *atpD* haplotypes obtained in this study and the ones obtained from GenBank (Stated below). On the trees, only the bootstrap values greater than 50 % are shown. *R. leguminosarum*^{T} (USDA 2370) AJ294405; *R. etli*^{T} (CFN42) AJ294404 (Gaunt et al 2001); ATCC 14480 EF113150; *R. pisi*^{T} (DSM 30132) EF113149; *R. phaseoli*^{T} (ATCC 14482) EF113151 (Santillana et al. 2008; Ramirez-Bahena et al. 2008); CCBAU 43229 GQ323589 (Tian et al. 2010); *R. fabae*^{T} (CCBA U33202) EF579929 (Tian et al. 2008); *R. huautlense*^{T} (SO2) AY688589; *R. yanglingense*^{T} (SH22623) AY907373; *R. mongolense*^{T} (USDA 1844) AY907372 (Vinuesa et al. 2005b); *R. galegae*^{T} (USDA 4128) AM418779 (Martens et al. 2008); *R. gallicum*^{T} (R6002sp) HM142762 (Lopez-Lopez et al. 2010); *R. lusitanum*^{T} (p1-7) DQ431671 (Valverde et al. 2006); *R. giardinii*^{T} HQ394216 (Robledo et al. 2011)

However, isolate CTG-412 appeared as sister taxon to the monophyletic group above with a 95 % bootstrap value in the NJ tree. The nucleotide similarities between CTG-412 and other haplotypes in this lineage were determined as 98.3 % (among CTG-412 and CFN42), 98.3 % (among CTG-412 and *R. gallicum* type strain), 98.1 % (among CTG-412 and isolate ICMP2672), and 98.5 % (among CTG-412 and other haplotypes determined in this study). MP analyses resulted in eight most parsimonious trees (length: 791 steps; CI: 0.764; RI: 0.735). In the MP tree (Fig. 7b), our *nodA* sequences also grouped with the same isolates (haplotypes) as in the NJ tree.

As representative for nitrogen fixation genes, we partially sequenced the *nifH* genes of our isolates. Primer sets nifHF/nifHI (Laguerre et al. 2001) and nifH1/nifH2 (Eardly et al. 1992) were used for amplifications and DNA sequencings of isolates CTG-403, CTG-407, and CTG-430 and isolates CTG-412, CTG-416, CTG-419, CTG-423, and CTG-427, respectively. The phylogenetic analysis of our *nifH* haplotypes, together with the ones from GenBank (stated in the legend of Fig. 8a, b) were carried out over 366 aligned nucleotides with 131 variable sites. Because the highest bootstrap values for NJ tree were determined with the TIM3ef+G (G: 0.407) substitution model, the NJ tree drawn with this model has been given (Fig. 8a). In MP analyses, we had a single most parsimonious tree (length: 210 steps; CI: 0.786; RI: 0.634) (Fig. 8b). Although they belong to different rhizobial species, six of our haplotypes obtained in this study (CTG-403, -407, -419, -423, -427, -430) showed the same nucleotide sequence with each other and the Meso-America-originated *R. etli* bv. *phaseoli* isolates: CFN42 (*R. etli* type strain), Olivia4, and CIAT 652. And our two other haplotypes, CTG-412 and CTG-416, showed the same nucleotide sequence with each other and appeared as closest taxa to the isolates above in the phylogenetic trees with a

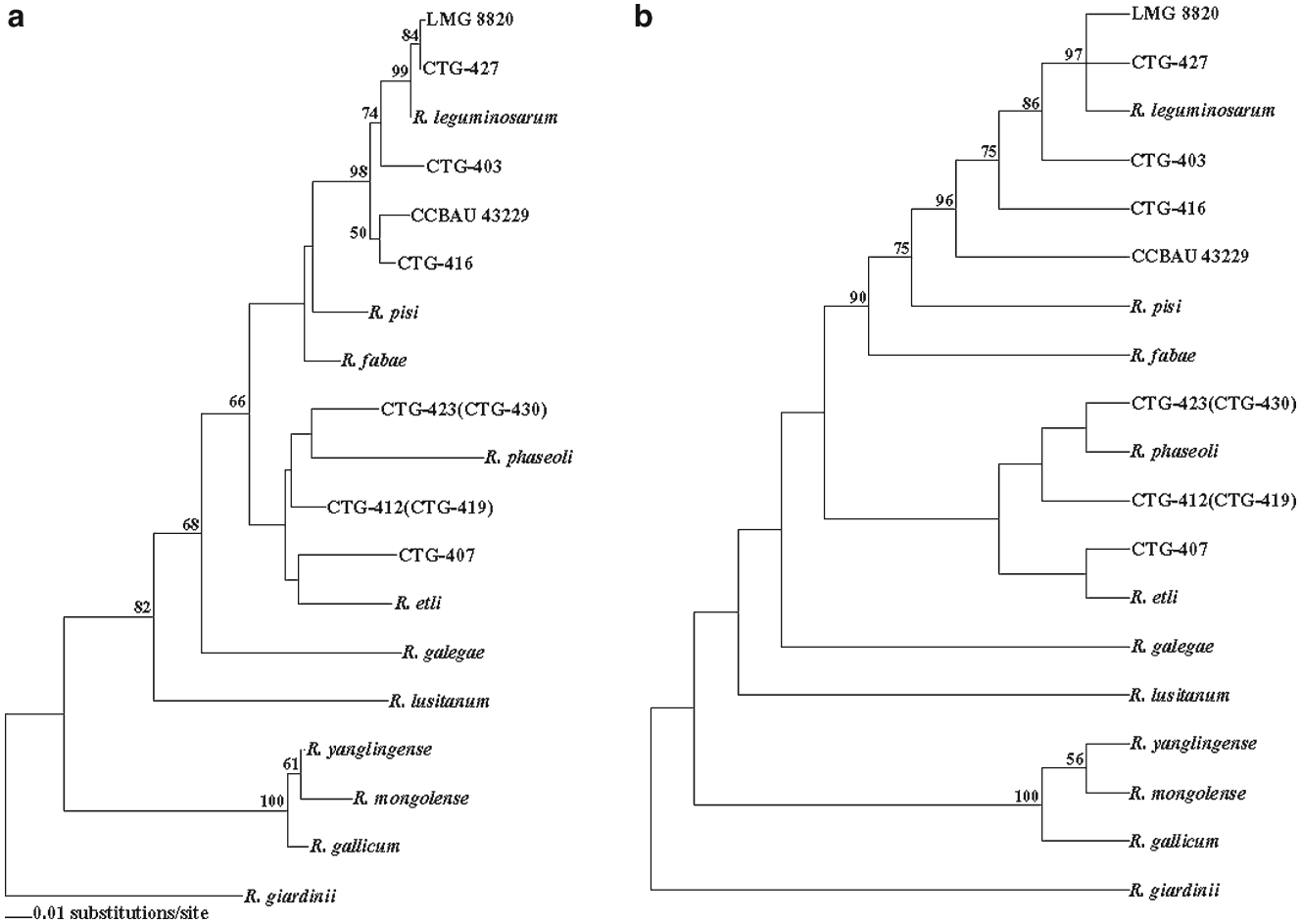

Fig. 6 NJ (**a**) and MP (**b**) trees showing the phylogenetic relationships of *glnII* haplotypes obtained in this study and the ones obtained from GenBank (stated below). On the trees, only the bootstrap values greater than 50 % are shown. *R. leguminosarum*^{T} (USDA 2370) AF169586; *R. galegae*^{T} (USDA 4128) AF169587 (Turner and Young 2000); *R. etli*^{T} (CFN42) CP000133.1 (Gonzalez et al. 2006); *R. pisi*^{T} (DSM 30132) JN580715; *R. phaseoli*^{T} (ATCC 14482) JN580716 (Aserse et al. 2012); CCBAU 43229 GQ323628 (Tian et al. 2010); *R. fabae*^{T} (CCBAU 33202) EF579935 (Tian et al. 2008); *R. yanglingense*^{T} (SH22623) AY929462; *R. mongolense*^{T} (USDA 1844) AY929453 (Silva et al. 2005); *R. gallicum*^{T} (R6002sp) AF529015 (Silva et al. 2003); *R. giardinii*^{T} EU488778 (Ribeiro et al. 2009); LMG 8820 FJ392869 (Han et al., unpublished); *R. lusitanum*^{T} (p1-7) EF639841 (Han et al., unpublished)

98.3 % nucleotide similarity. In the NJ tree, our haplotypes obtained in this study, the *R. etli*-related isolates from the Americas (CFN42, Olivia4, CIAT 652), and *R. gallicum* (*Phaseolus* spp. nodulating species from France) formed a monophyletic group suggesting a common history, and *R. tropici* (*Phaseolus* spp. nodulating species from Americas) appeared as sister taxon to this lineage with a 69 % bootstrap value. MP analyses also relatively supported this finding with a 85 % bootstrap value.

Discussion

Here, we analyzed a rhizobial collection comprised of 30 isolates obtained from *P. vulgaris* root nodules grown in four different cities in the Middle Black Sea region of Turkey (Fig. 1). We identified three rhizobial groups which are related to *Rhizobium leguminosarum* bv. *phaseoli* (*n*: 16), *R. phaseoli* (*n*: 6), and also a taxonomically uncertain group (*n*: 8) based on ARDRA and 16S rDNA *recA*, *atpD* and *glnII* nucleotide sequence phylogenies.

We used ARDRA for pre-grouping of the isolates with the restriction enzymes *Hinf*I, *Msp*I, *Nde*II and *Cfo*I, as suggested by Laguerre et al. (1994). Although this set seemed to be sufficient to discriminate between rhizobial species (including *R. etli* and *R. phaseoli*) when we theorically digested the type strains, it failed to distinguish our uncertain (isolates grouped with *R. etli* in 16S rDNA phylogeny) isolates that had a pattern similar to *R. phaseoli* because of a substitution at a *Cfo*I recognition site. Thus, we additionally digested our samples with *Pst*I to discriminate between the two groups, suggesting that in any study

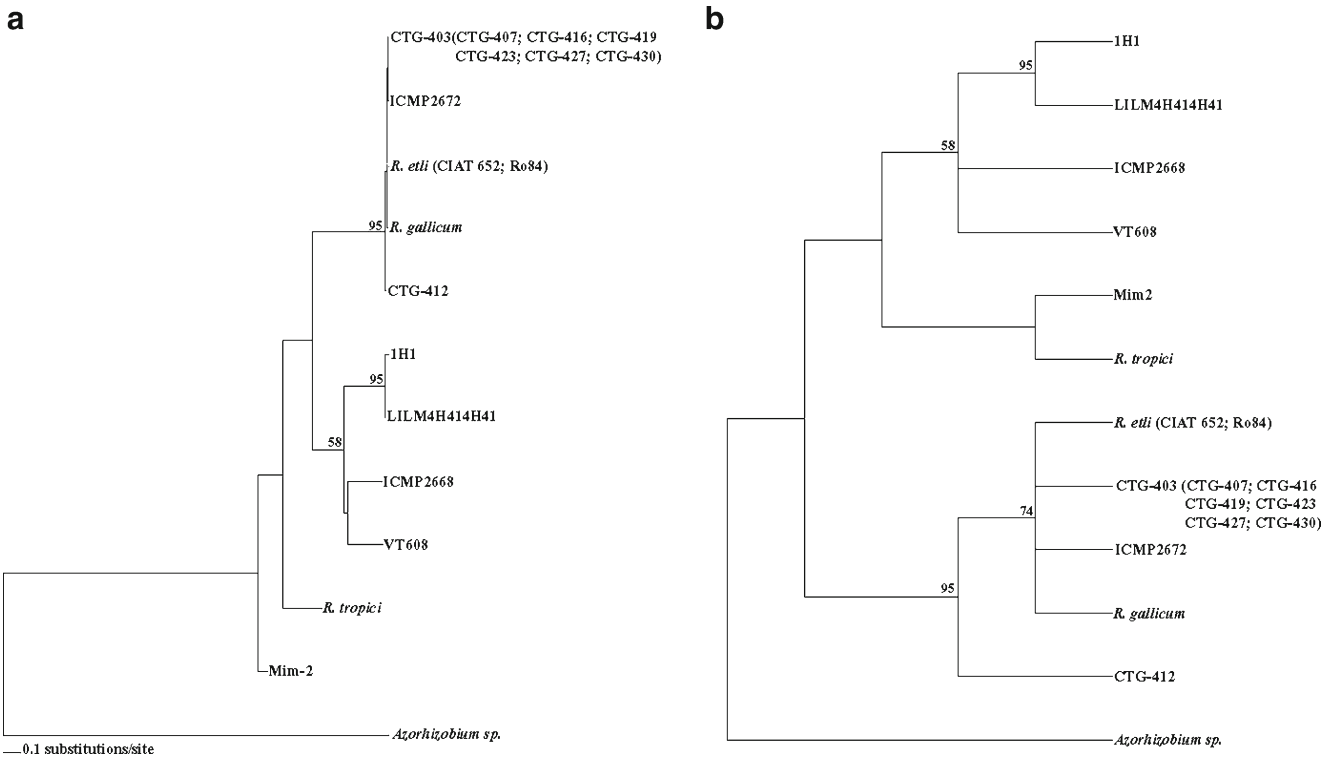

Fig. 7 NJ (**a**) and MP (**b**) trees showing the phylogenetic relationships of *nodA* haplotypes obtained in this study and the ones obtained from GenBank (stated below). On the trees, only the bootstrap values greater than 50 % are shown. *R. etli*^{T} (CFN42) NC_004041 (Gonzalez et al. 2003); ICMP2668 DQ100412; ICMP2672 DQ100403 (Weir 2006); 1H1 FJ800057; LILM4H4H41 FJ800056 (Mnasri et al. 2009); *R. tropici*^{T} (CFN299) X98514 (Debelle et al. 1996); Mim2 EU386136 (Elliott et al. 2009). CIAT 652 CP001076 (Gonzalez et al., unpublished); *R. gallicum*^{T} (PhD12) AJ300237; Ro84 AJ300239; *Azorhizobium* spp. AJ300261 (Moulin, unpublished); VT608 FJ715818 (Kim et al., unpublished)

involving a rhizobial collection, especially isolated from *P. vulgaris*, *Pst*I should be added to the restriction enzyme set of Laguerre et al (1994), or in addition to ARDRA, extra pre-grouping methods, such as RAPD, should be used.

Isolates CTG-409 and CTG-412 grouped with the *Rhizobium phaseoli* type strain ATCC 14482 in 16S rDNA, *recA* and *glnII* phylogenies and also showed significant nucleotide similarities with the type strain, while the only exception appeared in the *atpD* phylogeny which is obviously the result of a lateral transfer from a *R. leguminosarum*-related isolate. In this case, we can say that these two isolates and the ones sharing the same ARDRA pattern (CTG-410, -411, -418, -419) are related to *R. phaseoli*. We isolated all our *R. phaseoli*-related samples from a geographicaly homogenous, relatively narrow, long (about 200 km) and sea level (average altitude 15–30 m) corridor comprised of the seaside districts of Samsun (*n*: 4) and Sinop (*n*: 2) cities (Fig. 1; Table 1). Interestingly, samples collected from sites in the high altitude districts of the same cities had no *R. phaseoli*-related isolates.

Isolates CTG-423 and CTG-430 appeared to be related to *Rhizobium etli* in 16S rDNA nucleotide sequence phylogeny, but on the other hand these isolates showed *recA* and *glnII* haplotypes similar to *R. phaseoli* (Figs. 3a, b, 4a, b, 6a, b) and the *atpD* haplotype between *R. leguminosarum* and *R. fabae–R.pisi* (Fig. 5a, b). That is why these isolates and also the ones showing the same ARDRA pattern (CTG-417, -420, -421, -422, -424, -429) were named as a taxonomically uncertain group. But most of the genes analyzed in this study indicate that these isolates are related to the exogenic species *R. etli* or *R. phaseoli* rather than *R. leguminosarum*. These uncertain isolates were obtained from samples collected from Sinop (*n*: 6) and Amasya (*n*: 2) cities, but plant samples from Samsun and Ordu did not have this group. Recent studies have indicated that *R. etli* bv. *phaseoli* co-evolved with the common bean in Meso- and South America and was introduced to Europe after the discovery of the Americas (Columbus era, 1492), possibly as bean seeds as it has been shown that *R. etli* isolates can be carried on *P. vulgaris* seed testa (Perez-Ramirez et al. 1998; Aguilar et al. 2004). *P. vulgaris* seeds were imported to Turkey much later, about 250–300 years ago (Şehirali 1988). Thus, non-native uncertain (*R. etli*- or *R. phaseoli*-related) isolates obtained in this study might have been carried with the seeds to the Black Sea part of Turkey by a maritime route within the last few hundred years from

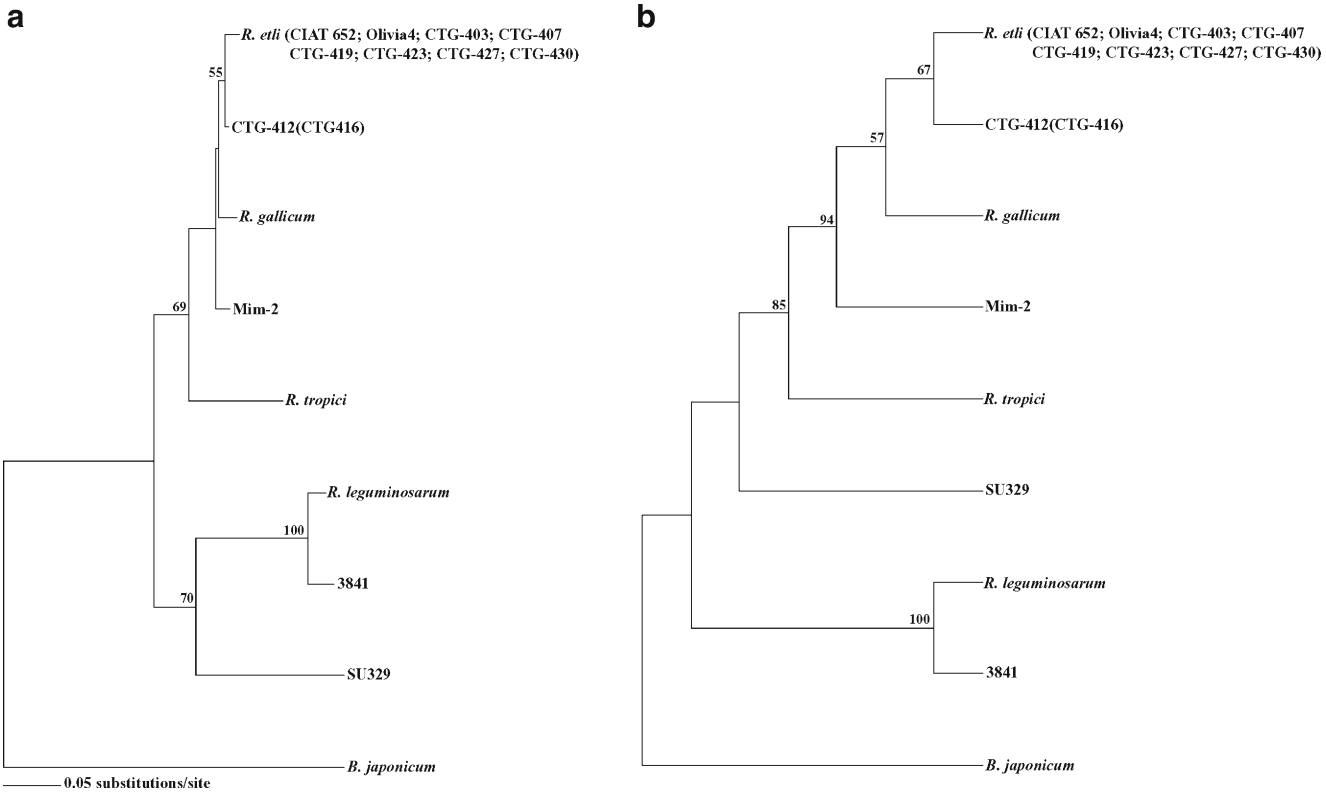

Fig. 8 NJ (**a**) and MP (**b**) trees showing the phylogenetic relationships of *nifH* haplotypes obtained in this study and the ones obtained from GenBank (stated below). On the trees, only the bootstrap values greater than 50 % are shown. *R. etli*^{T} (CFN42) U80928 (Ramirez-Romero et al. 1997); *R. tropici*^{T} (CIAT 899) M55225; Olivia4 M55227 (Eardly et al. 1992); *R. gallicum*^{T} (R6002sp) AF218126 (Laguerre et al. 2001); Mim-2 AF107621 (Wang et al. 1999); *R. leguminosarum*^{T} (USDA 2370) DQ450935 (Laranjo et al. 2008); 3841AM236084 (Young et al. 2006); SU329 K00490 (Scott et al. 1983); *B. japonicum* (USDA 110) K01620 (Fuhrmann and Hennecke 1984); CIAT 652 CP001076 (Gonzalez et al., unpublished)

European or African countries where *R. etli* isolates occurred, including Spain (Herrera-Cervera et al. 1999), France (Laguerre et al. 1993), Austria (Sessitsch et al. 1997), Jordan (Tamimi and Young 2004), Egypt (Shamseldin and Werner 2007), Ethiopia (Beyene et al. 2004), Tunusia (Mhamdi et al. 1999, 2002), Kenya (Anyango et al. 1995), Senegal, and Gambia (Diouf et al. 2000), or from the Americas where *R. phaseoli* isolates have been reported. Besides their ecological needs, this relatively short history in Anatolia may explain why exogenic species (uncertain isolates and *R. phaseoli*-related isolates) are distributed in a limited area around historical seaports and trade cities like Samsun and Sinop from where they started to spread across Anatolia, except for CTG-429 and CTG-430 from Amasya city. Of the two isolates, CTG-430 (the one selected for nucleotide sequencings) showed exactly the same 16S rDNA, *recA*, *atpD*, *glnII*, *nodA*, and *nifH* haplotypes as CTG-423, an isolate from Duragan (one of the inner districts of Sinop city; Fig. 1), indicating that these two isolates from Amasya were likely transfered to this city from Duragan. This geographical distribution can also suggest that some edaphic factors (e.g., soil temperature, salinity, or pH) might play a major role in the distribution of the rhizobial species, as reported in previous studies. Martinez-Romero et al (1991) identified *P. vulgaris* and *Leucaena* nodulating *R. tropici* as a high temperature and acidity tolerating species. Anyango et al. (1995) also reported *R. tropici* as the major rhizobial symbiont of *P. vulgaris* grown in acid soils (pH 4.5) of Kenya, due to its broad range ecological valence for these factors. Thus, the limited geographical distribution of exotic (uncertain group and *R. phaseoli*-related isolates) species obtained in this study might be the result of such ecological factors. Further studies on the ecological needs of rhizobial symbionts will provide an insight into this point and will help with producing effective natural nitrogen fertilizers as alternatives to the chemical ones which cause much ecological damage.

16S rDNA phylogenies have shown the relationsships of isolates CTG-403, CTG-407, CTG-416, and CTG-427 with *R. leguminosarum*, and *glnII* phylogeny mostly supported this finding with the exception of CTG-407, which showed a *glnII* haplotype similar to *R. etli*. On the other hand, in *recA* phylogen,y we determined some lateral transfer events for isolates CTG-407 and CTG-416. And in *atpD* phylogeny,

these isolates were mostly placed (except CTG-427 that showed a close relationship with USDA 2370) between *R. leguminosarum* and the *R. fabae–R. pisi* clade. All these results demonstrate that these isolates, and also the ones showing the same ARDRA pattern, are related to *R. leguminosarum* but have had some lateral gene transfers with other rhizobial species. As a result, all the isolates from Ordu (n = 8), those from the high altitude provinces of Samsun (n = 4), and four isolates from Amasya were identified as *R. leguminosarum*. This distribution of the isolates indicates that *R. leguminosarum* bv. *phaseoli* is the predominant species nodulating *P. vulgaris* in the Middle Blacksea part of Turkey.

We used *recA*, *atpD* and *glnII* as housekeeping genes to verify our identification with 16S rDNA. There have been various studies indicating some phylogenetic congruences between 16S rDNA and other housekeeping genes in rhizobia and bacteria in general (Eisen 1995; Gaunt et al. 2001). However, we determined a many incongruences between the genes analyzed. Two explanations regarding this incongruence can be proposed: (1) these genes might have evolved at different evolutionary rates in these isolates, or (2) there might have been lateral transfers of these genes between different rhizobial species. The fact that most of the earlier studies have shown obvious phylogenetic congruences makes the first explanation less likely (Eisen 1995; Gaunt et al. 2001). On the other hand, there have been examples of lateral transfers of genes (i.e. 16S rDNA) between different rhizobial species (Sullivan et al. 1996), suggesting that lateral transfer is the more likely explanation of the inconcruence between these genes. According to our results, it is obvious that lateral gene transfers between *R. leguminosarum*, *R. etli* and *R. phaseoli* isolates that share the same ecological niches (same host plant, rhizosphere, etc.) are common in nature, which indicates that the genetic barriers among these species may not be enough to characterize them as true species. In conclusion, our results indicate that the rhizobial lineage containing *R. leguminosarum*, *R. trifolii*, *R. etli*, *R. phaseoli* and *R. fabae* should be reconsidered in the context of the boundaries of these species, and also that our results are further evidence that more than one gene should be used for the identification of rhizobial species.

For the characterization of sym plasmides that our isolates harbor, we used *nodA* (codes for an acyltransferase) and *nifH* (structural gene of dinitrogenase reductase) genes as representatives for nodulation and nitrogen fixation genes, respectively. For amplification of *nodA*, we initially used primers nodA1/nodA2 (Haukka et al. 1998) that amplify a 666-bp-long fragment between the 14th base of *nodA* and the 88th base of *nodB*, but we could not get an appropriate sized band. When we used primer nodA3 (Zhang et al. 2000) as reverse primer that anneals an inner position of *nodA*, we got the appropriate sized (approx. 600 bp) band. These results suggested the possibility that the *nodA* gene may not be followed by *nodB* in our isolates, but they may be in seperate operons as previously reported from the *R. etli* type strain (CFN42) sym plasmide (p42) (Vazquez et al. 1991). But this assumption needs examination. In both the NJ and MP trees (Fig. 5a, b), all our *nodA* sequences from different species (*R. leguminosarum*, *R. phaseoli* and the uncertain group) also grouped with *R. etli*-related isolates. Both indirect evidence from the organization of nod operon and direct evidence from phylogenetic analyses of the *nodA* gene indicate a p42 sym plasmide in all Turkish *P. vulgaris* rhizobium isolates. *NifH* is the most common gene used as a marker for nitrogen fixation genes. So far, there have been several studies involving the phylogenetical evaluation of the *nifH* genes of rhizobial species. Some have suggested that, although *nifH* is mostly an accessory gene (carried on a pSym in fast-growing rhizobia), it phylogenetically follows the chromosomal 16S rDNA gene and shares a similar evolutionary history with it (Hennecke et al. 1985; Dobert et al. 1994). On the other hand, others have presented opposite results indicating coevolution of *nifH* with other sym plasmid-borne genes like *nodA* and *nodC* instead of chromosomal 16S rDNA (Haukka et al. 1998; Laguerre et al. 2001). Six of the eight rhizobial isolates collected in this study showed an exactly identical *nifH* haplotype with CFN42 (and also with two other Meso-America-originated *R. etli* bv. *phaseoli* isolates, CIAT 652 and Olivia4). Although the two other isolates, CTG-412 and CTG-416, showed a different *nifH* haplotype, they were grouped within the same lineage with the other isolates collected in this study and also with CFN42 (Fig. 6a, b). The results of our phylogenetic analysis clearly support the second hypothesis, assuming that *nifH* coevolves with the other sym plasmid-borne genes but not with chromosomal genes. In addition to *nodA*, our *nifH* sequence phylogenies also indicated that all *P. vulgaris* related isolates obtained in our study likely have a p42 sym plazmid, even those that are related to *R. phaseoli* and *R. leguminosarum*.

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