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ORIGINAL ARTICLE

Genetic diversity of *Vicia faba* L. and *Pisum sativum* L. nodulating rhizobia in the central Black Sea region of Turkey

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Abstract In this study, we obtained a total of 60 rhizobial isolates from root nodules of *Vicia faba* L. ($n = 30$) and *Pisum sativum* L. ($n = 30$) grown in the Central Black Sea region of Turkey. The 16S rDNA PCR-RFLP analysis with enzymes *Cfo*I, *Hinf*I, *Nde*II and *Msp*I revealed a single pattern. Moreover, nucleotide sequence phylogenies based on both the 16S rDNA and *recA* suggested that these isolates belonged to *Rhizobium leguminosarum*. Phylogenetic analysis showed that some of our *V. faba* L.-originated isolates were closely related, indicating molecular evidence for the selection of some special *R. leguminosarum* bv. *viciae* isolates by *V. faba* L., as suggested in previous studies. Network analysis based on *recA* sequences revealed a common evolutionary history for Turkish, European, North and South American, and Jordanian *R. leguminosarum* bv. *viciae* isolates. We isolated four haplotypes using *nodA* and *nifH* nucleotide sequence data, i.e. four types of sym plasmids. Two of these types were common to rhizobial isolates from both *V. faba* L. and *P. sativum* L., indicating that nodulation factors may not be the mechanism for selection of the special *R. leguminosarum* bv. *viciae* populations by *V. faba* L.

Keywords Rhizobium · *V. faba* · *P. sativum* · Phylogeny · Nodulation

Introduction

Faba bean (*Vicia faba* L.) is used as a major food and feed legume because of the nutritional value of its seeds which contain high amounts of protein and starch; some varieties of this plant are also rich in L-DOPA (3,4-dihydroxy-phenylalanine) which is used in the treatment of Parkinson's disease (Van Berkum et al. 1995; Duc et al. 2010). Recent findings have showed that faba bean was commonly used in the late 10th millennium B.P., suggesting that domestication of this plant might have occurred much earlier than previously supposed and that it should also be considered one of the founder crops cultivated in ancient times (Tanno and Willcox 2006). Because of its failure to cross-pollinate with other *Vicia* spp., and since no wild faba bean has ever been recorded in nature, the ancestry (progenitor) of this plant is still a mystery (Muratova 1931; Duc et al. 2010). However, several studies have suggested different origins for faba bean, such as south-eastern Europe and south-western Asia (Muratova 1931; Maxted 1995), central Asia (Ladizinsky 1975), and the Near East, with four different dissemination routes: (1) to Europe; (2) along the North Africa coast to Spain; (3) along the Nile to Ethiopia; and (4) from Mesopotamia to India (Cubero 1973; Cubero 1974). The other host, pea (*Pisum sativum* L.), examined in this study has been grown for many centuries as an important source of animal and human food with many varieties including field pea, market pea and dried pea, and it is considered one of the founder crops like faba bean. Abyssinia and Afghanistan have been suggested as possible centres of origins for the pea. It was probably brought to the Mediterranean basin later. The pea might have spread to other parts of Europe and Asia from all these possible source areas (Cousin 1997; Abbo et al. 2005). Because of its transcontinental position, Turkey might have played a major role as a potential centre of origin or at least a pathway for dissemination of both faba bean and pea to the rest of the world.

Rhizobium leguminosarum bv. *viciae* (*Rlv*) is the specific symbiont of the legumes of the tribe Vicieae, comprising the genera *Vicia*, *Pisum*, *Lens* and *Lathyrus* (Laguerre et al. 2003). To date, *Rlv* isolates identified using current

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moleculer methods have been reported from faba bean root nodules in China (Tian et al. 2007; Kan et al. 2007; Han et al. 2008a; Hou et al. 2009), Peru (Santillana et al. 2008), Jordan, UK (Mutch et al. 2003), Korea (Kwon et al. 2005), Egypt (Shamseldin et al. 2009) and Italy (Moschetti et al. 2005), and from pea root nodules in the USA (Van Berkum et al. 1996), Peru (Santillana et al. 2008), Korea (Kwon et al. 2005) and India (Rahi et al. 2012). In addition to *Rlv*, other rhizobial species have been reported as microsymbionts, such as *Rhizobium etli* for faba bean and pea and *Sinorhizobium meliloti* for faba bean (Tian et al. 2007; Santillana et al. 2008; Shamseldin et al. 2009)

Although faba bean and pea are important crops for Turkey, to the author's knowledge there are no reports focusing on the genetic diversity of rhizobia nodulating these host plants in that country. Thus, the current study was conducted to make inferences about the contribution of Turkey as a source area and dissemination route for rhizobia isolates nodulating faba bean and pea, by using some core and extra chromosomal gene sequences obtained from rhizobia isolated from faba bean and pea root nodules cultivated in the central Black Sea region of Turkey.

Materıals and methods

Bacterial isolates

In this study, 30 different sites located in the central Black Sea region of Turkey (Samsun, Sinop, Ordu and Amasya cities) were selected as sampling areas (Table 1). Non-certified local *V. faba* L. (Sakiz type) and *P. sativum* L. (Sultani type) plants were collected as pairs (0–2 m distant from each other) from the same fields at each site to reveal the possible host–symbiont specificity (*R. leguminosarum* bv. *viciae* populations specific to *V. faba* L.). Samplings was done during April and May 2008. The method of Vincent (1970) was used to isolate rhizobial samples from active (pink-coloured) faba bean and pea root nodules. Yeast extract mannitol agar (YMA) medium (Vincent 1970) was used for isolations and purifications of rhizobial isolates. Growth on peptone glucose agar (PGA-with brom cresol purple), colony morphology on YMA medium (with brom thimol blue) and microscopic examination by Gram-staining tests were used to see whether isolates were pure or contaminated (Vincent 1970; Somasegaran and Hoben 1985; Pollack et al. 2002; Kuykendall et al. 2005). To test the nodulation and nitrogen fixation capacity of our isolates, authentication tests were used following the method of Vincent (1970), with three replicates for each isolate. After 4 weeks of incubation in a growth chamber in 14 h light and 10 h dark, isolates were evaluated for their nodulation ability and symbiotic efficiency.

Molecular analysis

Genomic DNA extractions from rhizobia were made with the CTAB/NaCl miniprep method (Temizkan and Arda 2004) using 2 ml fresh bacterial cultures grown in TY (Tryptone Yeast Extract) broth media (Ditta et al. 1987). Genomic DNA was stored at −20 °C prior to use.

To screen the genetic diversity amongst isolates, 16S rDNA PCR-RFLP (Restriction Fragment Length Polymorphism) analysis was employed, using restriction enzymes *Hinf*I (New England BioLabs), *Msp*I (Fermentas), *Nde*II (Promega) and *Cfo*I (Promega), as reported by Laguerre et al. (1994). Primers fD1 and rD1 (Weisburg et al. 1991) were used to amplify the 16S rDNA region for RFLP analysis, using the PCR conditions given in Table 2. PCR products were purified using the QIAquick PCR Purification Kit. All restriction reactions were done in a 10 µl volume, as specified by the manufacturer, and digested bands were separated in 2.5 % metaphore agarose gel (Lonza, USA) prepared in 1X TBE (Tris-Borate-EDTA) buffer.

Identifications of our isolates were made with the nucleotide sequence phylogenies of two chromosomal genes, 16S rDNA and *recA* (gene coding for recombinase A protein). Amplifications of 16S rDNA for nucleotide sequencing were made with two sets of primers, fD1/rD1 (Weisburg et al. 1991) and an internal set pA/pF (Zhang et al. 1999) for a more reliable sequencing. For amplification of *recA*, the primer set recA-For/recA-Rev (Gaunt et al. 2001) was used. To characterise the symbiotic elements, the *nodA* and *nifH* genes coding for acyltransferase and dinitrogenase reductase, respectively, were analysed. Amplifications of *nodA* were done with the primers nodA1 and nodA2 (Haukka et al. 1998). For *nifH* amplification, we used the primers nifHctg (designed in this study, 5′- CTC ATC GTC GGC TGT GAC CC -3′) and nifHI (Laguerre et al. 2001). The primer set oMP199/oMP196 (Ovtsyna et al. 1999) was used to check if the other nodulation gene, that is *nodX* coding for *O*-acetyl transferase, was present in our isolates. PCR protocols used for 16S rDNA, *recA*, *nodA*, *nifH* and *nodX* amplifications are given in Table 2. For all amplifications, 50 µl PCR mixtures were prepared as follows; template DNA <0.5 µg, 1.5 mM $MgCl_2$, 1.25 U Taq polymerase (Promega, Go-Taq Flexi DNA Polymerase), 0.8 mM dNTP mix (Amresco), 1X PCR buffer (Go-Taq Green Buffer; Promega), 0.4 pmol of each primer in final concentration (0.6 pmol used for primers nifHctg/nifHI) and ddH_2O. The PCR products were electrophoresed in 1 % agarose gel (Amresco, Solon, OH, USA) prepared in 1X TBE (Tris-Borate-EDTA) buffer. When extra bands appeared on the gel, the appropriate-sized nucleotide band was removed from the gel and kept in a microcentrifuge tube containing 50 µl ddH_2O for 1 h and 10 µl of the water was used as a template for the next PCR reaction (Haukka et al. 1998). All

Table 1 Codes, geographical origins and accession numbers for nucleotide sequences (16S rDNA, *recA*, *nodA* and *nifH* genes) of rhizobium isolates collected from four provinces in the central Black Sea region of Turkey

Origin	*V. faba* isolates	Nucleotide sequence accession number for *V. faba* isolates				*P. sativum* isolates	Nucleotide sequence accession number for *P. sativum* isolates			
		16S rDNA	*recA*	*nodA*	*nifH*		16S rDNA	*recA*	*nodA*	*nifH*
ORDU-Centre	CTG-01Vf	–	–	–	–	CTG-01Ps	KC609472	KC609432	KC620568	KC609456
ORDU-Camas	CTG-02Vf	–	–	–	–	CTG-02Ps	–	–	–	–
ORDU-Cayiralan	CTG-03Vf	–	–	–	–	CTG-03Ps	KC609473	KC609433	KC620569	KC609457
ORDU-Tekkiraz	CTG-04Vf	–	–	–	–	CTG-04Ps	–	–	–	–
ORDU-Fatsa	CTG-05Vf	KC609464	KC609440	KC620560	KC609448	CTG-05Ps	–	–	–	–
ORDU-Unye	CTG-06Vf	–	–	–	–	CTG-06Ps	–	–	–	–
ORDU-Golkoy	CTG-07Vf	–	–	–	–	CTG-07Ps	–	–	–	–
ORDU-Mesudiye	CTG-08Vf	KC609471	KC609447	KC620567	KC609455	CTG-08Ps	–	–	–	–
SAMSUN-Center	CTG-09Vf	KC609465	KC609441	KC620561	KC609449	CTG-09Ps	–	–	–	–
SAMSUN-Vezirkopru	CTG-10Vf	KC609466	KC609442	KC620562	KC609450	CTG-10Ps	–	–	–	–
SAMSUN-Carsamba	CTG-11Vf	–	–	–	–	CTG-11Ps	–	–	–	–
SAMSUN-Terme	CTG-12Vf	–	–	–	–	CTG-12Ps	–	–	–	–
SAMSUN-Bafra	CTG-13Vf	–	–	–	–	CTG-13Ps	–	–	–	–
SAMSUN-Alacam	CTG-14Vf	–	–	–	–	CTG-14Ps	KC609477	KC609437	KC620573	KC609461
SAMSUN-Kavak	CTG-15Vf	–	–	–	–	CTG-15Ps	KC609475	KC609435	KC620571	KC609459
SAMSUN-Ladik	CTG-16Vf	–	–	–	–	CTG-16Ps	–	–	–	–
SİNOP-Boyabat	CTG-17Vf	KC609467	KC609443	KC620563	KC609451	CTG-17Ps	–	–	–	–
SİNOP-Duragan	CTG-18Vf	–	–	–	–	CTG-18Ps	–	–	–	–
SİNOP-Center	CTG-19Vf	–	–	–	–	CTG-19Ps	–	–	–	–
SİNOP-Erfelek	CTG-20Vf	KC609468	KC609444	KC620564	KC609452	CTG-20Ps	–	–	–	–
SİNOP-Gerze	CTG-21Vf	–	–	–	–	CTG-21Ps	–	–	–	–
SİNOP-Cove	CTG-22Vf	–	–	–	–	CTG-22Ps	KC609476	KC609436	KC620572	KC609460
SİNOP-Ayancik	CTG-23Vf	–	–	–	–	CTG-23Ps	KC609474	KC609434	KC620570	KC609458
SİNOP-Kepez	CTG-24Vf	–	–	–	–	CTG-24Ps	–	–	–	–
AMASYA-G. Hacikoy	CTG-25Vf	KC609469	KC609445	KC620565	KC609453	CTG-25Ps	KC609478	KC609438	KC620574	KC609462
AMASYA-Merzifon	CTG-26Vf	–	–	–	–	CTG-26Ps	–	–	–	–
AMASYA-Tasova	CTG-27Vf	KC609470	KC609446	KC620566	KC609454	CTG-27Ps	–	–	–	–
AMASYA-Center	CTG-28Vf	–	–	–	–	CTG-28Ps	KC609479	KC609439	KC620575	KC609463
AMASYA-Suluova	CTG-29Vf	–	–	–	–	CTG-29Ps	–	–	–	–
AMASYA-Goynucek	CTG-30Vf					CTG-30Ps	–	–	–	–

Table 2 PCR protocols and primers used in this study

Gene	Primer	ID	D	A	E	FE
16S rDNA	fD1/rD1[a]×35 Cycles	95 °C/5 min	95 °C/45 s	55 °C/45 s	72 °C/2 min	72 °C/4 min
16S rDNA	pA/pF[b]×35 Cycles	95 °C/3 min	95 °C/1 min	55 °C/1 min	72 °C/1 min	72 °C/5 min
recA	recA-For/recA-Rev[c]×30 Cycles	95 °C/5 min	95 °C/45 s	50 °C/1 min	72 °C/1 min	72 °C/2 min
nodA	nodA1/nodA2[d]×35 Cycles	95 °C/5 min	95 °C/45 s	49 °C/45 s	72 °C/45 s	72 °C/5 min
NifH	nifHI[e]/nifHctg[f]×40 Cycles	95 °C/3 min	94 °C/1 min	59 °C/1 min	72 °C/1 min	72 °C/5 min
nodX	oMP199/oMP196[g]×39 Cycles	95 °C/3 min	94 °C/1 min	48 °C/1 min	72 °C/1.5 min	72 °C/7 min

ID Initial denaturation, *D* Denaturation, *A* Annealing, *E* Extension, *FE* Final Extension

[a] Weisburg et al. 1991

[b] Zhang et al. 1999

[c] Gaunt et al. 2001

[d] Haukka et al. 1998

[e] Laguerre et al. 2001

[f] This study

[g] Ovtsyna et al. 1999

PCR amplifications in this study were made using a MGW-Biotech thermal cycler and the visualisations of electrophoresis gels (stained with ethidium bromide) were done with the GeneGenius Bio imaging system (Syngene; Synoptics Group, Cambridge, UK).

Nucleotide sequencings of 16S rDNA, *recA*, *nodA* and *nifH* genes were performed commercially (by Macrogen, Korea) using the sequencer Roche 454 GSFLX Titanium in both directions with the same primers used for PCR amplifications. All new sequences obtained in this study were deposited in the NCBI data bank under accession numbers KC609432–KC609479 and KC620560–KC620575 (Table 1). The SeqMan II module of the LASERGENE 99 system (Applied Biosystems) was used to assemble the nucleotide sequences that were copied from both strands. The multiple nucleotide sequence alignments of our new haplotypes, together with the ones obtained from GenBank (see figure captions), were generated using ClustalX (Thompson et al. 1997), and optimised by hand with BioEdit (Hall 1999). The Akaike information criterion (AIC) and Bayesian information criterion (BIC) tests were applied with the jModelTest v.0.1 package program (Guindon and Gascuel 2003; Posada 2008) to determine the optimal DNA substitution model for our datasets. To evaluate the evolutionary relationships among isolates, the Neighbor-Joining (NJ) (Saitou and Nei 1987) and Maximum-Likelihood (ML) methods were implemented in PAUP* v.4.0b10 (Swofford 1998) and PhyML 3.0 (Guindon and Gascuel 2003), respectively. The bootstrap tests (Efron 1982; Felsenstein 1985) for the NJ and ML trees were applied on 10,000 and 1,000 pseudoreplicates, respectively, with the same substitution models and software programs used for phylogenies. Additionally, the consensus bootstrap tree for ML was obtained using the Consense tool of PHILIP v.3.68 (Felsenstein 2004). For visualisation of the relationships within *recA* haplotypes of *R. leguminosarum*, we calculated the median-joining network (Bandelt et al. 1999) included in Network 4.5.1.2 (www.fluxus-engineering.com). This method identifies groups of haplotypes and introduces hypothetical (non-observed) haplotypes to construct the parsimony network. In cases where there are shallow divergence datasets, there are advantages in using a median-joining network to depict relationships (Posada and Crandall 2001), and simulation studies have demonstrated that this method provides reliable estimates of the true genealogy (Cassens et al. 2005; Woolley et al. 2008).

Results

Isolates and conventional tests

In this study, we isolated a total of 60 rhizobial samples from *V. faba* L. (30 isolates) and *P. sativum* L. (30 isolates) root nodules collected from four different provinces (Samsun, Ordu, Sinop and Amasya) in the cental Black Sea region of Turkey (Table 1). All of these isolates were Gram-negative, rod-shaped cells and they neither grew on PGA nor changed the pH of that media. All isolates showed the typical morphological characteristics of rhizobia and were not contaminated. At the end of the 4 week incubation period, all our rhizobium isolates formed pink, indeterminate active root nodules on their original hosts (*V. faba* L. or *P. sativum* L.), suggesting that they harbour a pSym suitable for Viciae tribe plants.

PCR-RFLP analysis of the 16S rDNA gene

RFLP analysis of 16S rDNA using *Cfo*I, *Hinf*I, *Msp*I and *Nde*II enzymes revealed 7 (approx. 320, 280, 280, 170, 135,

115, 100 bp), 3 (approx. 1150, 200, 100 bp), 5 (approx. 500, 400, 220, 160, 120 bp) and 6 (700, 250, 180, 180, 80, 60 bp) digestion bands, respectively, for all isolates obtained in this study (Fig. 1). Because all isolates showed the same 16S rDNA-RFLP pattern, they were assumed to be the same species. Thus, we chose a total of 16 representative isolates from different sites and host plants for 16S rDNA, *recA*, *nodA* and *nifH* nucleotide sequencings (Table 1).

DNA sequencings and phylogenetic analysis

Approximately 1,250 bp of the 16S rDNA gene were sequenced for 16 representative rhizobial isolates (Table 1). Phylogenetic analysis of our new 16S rDNA sequences and the rhizobial type strains downloaded from GenBank (see the caption to Fig. 2) were conducted by using 1,210 aligned nucleotides having 122 polymorphic sites. For our dataset, AIC and BIC tests proposed TIM2+I+G (I: 0.625; G: 0.183) and HKY+I (I: 0.88) substitution models, respectively. However, we presented the NJ and ML trees produced using the TIM2+I+G model because with this model they showed the highest bootstrap values. In both the NJ and ML trees, all our isolates formed a monophyletic group with *R. leguminosarum* isolates USDA 2370 (type strain) and ATCC 14480 (bv. *trifolii*). Eight of our isolates (CTG-01Ps, -03Ps, -14Ps, -15Ps, -22Ps, -25Ps, -08Vf, -25Vf) showed the same 16S rDNA haplotype with ATCC 14480. Additionally, pairwise nucleotide similarities among all isolates within this monophyletic group were between 99.5 and 99.9 %. *Rhizobium phaseoli*, *Rhizobium indigoferae* and *Rhizobium pisi* appeared as the closest group to the *R. leguminosarum* lineage with a 54 % bootstrap value in the NJ tree.

Approximately 570 bp of the *recA* gene were sequenced for the selected rhizobial isolates (Table 1). Phylogenetic analysis was conducted on 396 aligned nucleotides having 135 polymorphic sites. Both AIC and BIC tests suggested TIM2+I+G (I: 0.572; G: 0.89) the nucleotide substitution model. In the NJ and ML trees, our isolates grouped in two main sublineages that seemed to be clearly related to *R. leguminosarum*. Most of our *V. faba* L. related rhizobial isolates (CTG-05Vf, -10Vf, -17Vf, -20Vf, -27Vf) and two *P. sativum* L. related isolates (CTG-01Ps and -28Ps) constituted the first sublineage with USDA 2370 (*R. leguminosarm* type strain) and isolate ATCC 14480 (*R. leguminosarm* bv. *trifolii*). The bootstrap values for the nodes within this sublineage were relatively high (Fig. 3) and the nucleotide sequence similarities were between 97.7

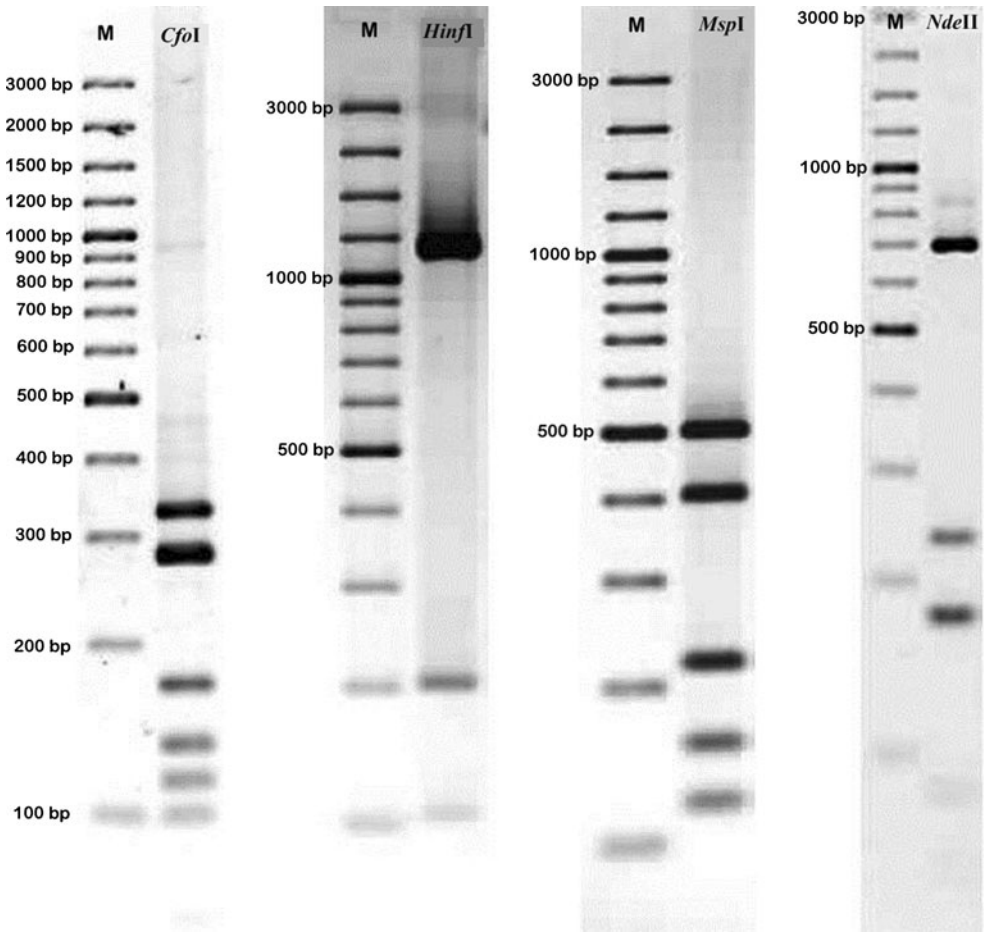

Fig. 1 16S rDNA PCR-RFLP patterns derived from digestions with the restriction enzymes *Cfo*I, *Hinf*I, *Msp*I and *Nde*II. M refers to DNA Marker (Fermentas, GeneRuler, 100 bp Plus DNA Ladder)

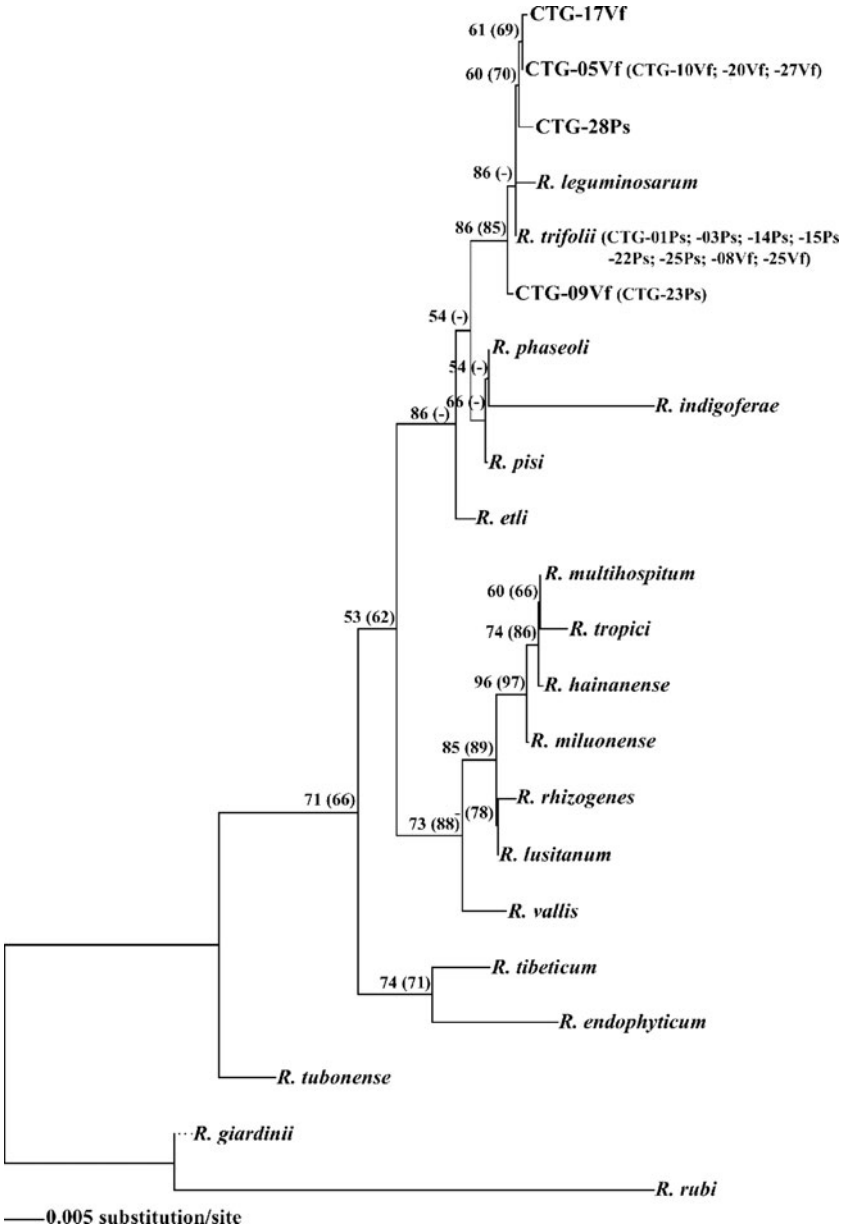

Fig. 2 NJ tree showing the phylogenetic relationships among 16S rDNA haplotypes obtained in this study and type strains of some rhizobial species obtained from GenBank (below). Bootstrap values of the ML tree produced with the same substitution model (TIM2+I+G) are given in *parentheses*. Bootstrap values greater then 50 % are shown. Rhizobial type strains and their accession numbers for 16S rDNA are as follow: *R. leguminosarum*^{T} U29386; *R. etli*T U28916; (Van Berkum et al. 1996); *R. hainanense*^{T} U71078 (Chen et al. 1997); *R. indigoferae*^{T} AF364068 (Wei et al. 2002); *R. rhizogenes*^{T} D01257; *R. rubi*^{T} D14503 (Sawada et al. 1993); *R. lusitanum*^{T} AY738130 (Valverde et al. 2006); *R. tropici*^{T} U89832 (Van Berkum et al. 1998); *R. pisi*^{T} AY509899; *R. phaseoli*^{T} EF141340; *R. trifolii* AY509900 (Ramirez-Bahena et al. 2008); *R. multihospitum*^{T} EF035074 (Han et al. 2008b); *R. miluonense*^{T} EF061096 (Gu et al. 2007); *R. vallis*^{T} FJ839677 (Wang et al. 2011); *R. tibeticum*^{T} EU256404 (Hou et al. 2009); *R. endophyticum*^{T} EU867317 (Lopez-Lopez et al. 2010); *R. tubonense*^{T} EU256434 (Zhang et al. 2011); *R. giardinii*^{T} U86344 (Amarger et al. 1997).

and 99.4 %. Separately, most of our *P. sativum* L. originated rhizobial isolates (CTG-03Ps, -14Ps, -15Ps, -22Ps, -23Ps, -25Ps) and three *V. faba* L. originated isolates (CTG-08Vf, -09Vf, -25Vf) formed the second lineage that appeared as sister to the first one and with a 93 % bootstrap value in the NJ tree. The nucleotide sequence similarities within this sublineage varied between 96.7 and 98.2 %. Overall, the *recA* nucleotide sequence similarities among the isolates for the whole *R. leguminosarum* lineage were between 96.7 and 99.4 %.

Nearly 570 bp of the *nodA* gene were sequenced for the selected isolates (Table 1). Phylogenetic analysis was carried out over 442 aligned nucleotides with 217 segregated sites. Both AIC and BIC tests suggested the HKY+G (G: 0.628) nucleotide substitution model. Both in the NJ and ML trees, *Rlv nodA* haplotypes grouped in four lineages (Fig. 4). Most of the haplotypes (*n*: 9) grouped in the first lineage. All our isolates, except for CTG-01Ps and CTG-28Ps, grouped in this lineage. The isolates CTG-10Vf, -17Vf, -27Vf (from *V. faba* L.), CTG-23Ps, -25Ps (from *P. sativum* L.) and isolates 248 (*V. faba* L. from the U.K.) and 3841 (*P. sativum* L. from the U.K.) had the same *nodA* haplotype. Isolate CTG-03Ps had a unique haplotype and it was sister to the first haplotype mentioned above, with 68 % and 67 % bootstrap values in the NJ and ML trees, respectively. Isolate CTG-22Ps had the same haplotype as FB9071 (*V. faba* L. from Tunisia) and grouped with the first two haplotypes with a 100 % bootstrap value in both the NJ and ML trees. Our other isolates (CTG-05Vf, -08Vf, -09Vf, -20Vf, -25Vf from *V. faba* L. and CTG-14Ps, -15Ps from *P.*

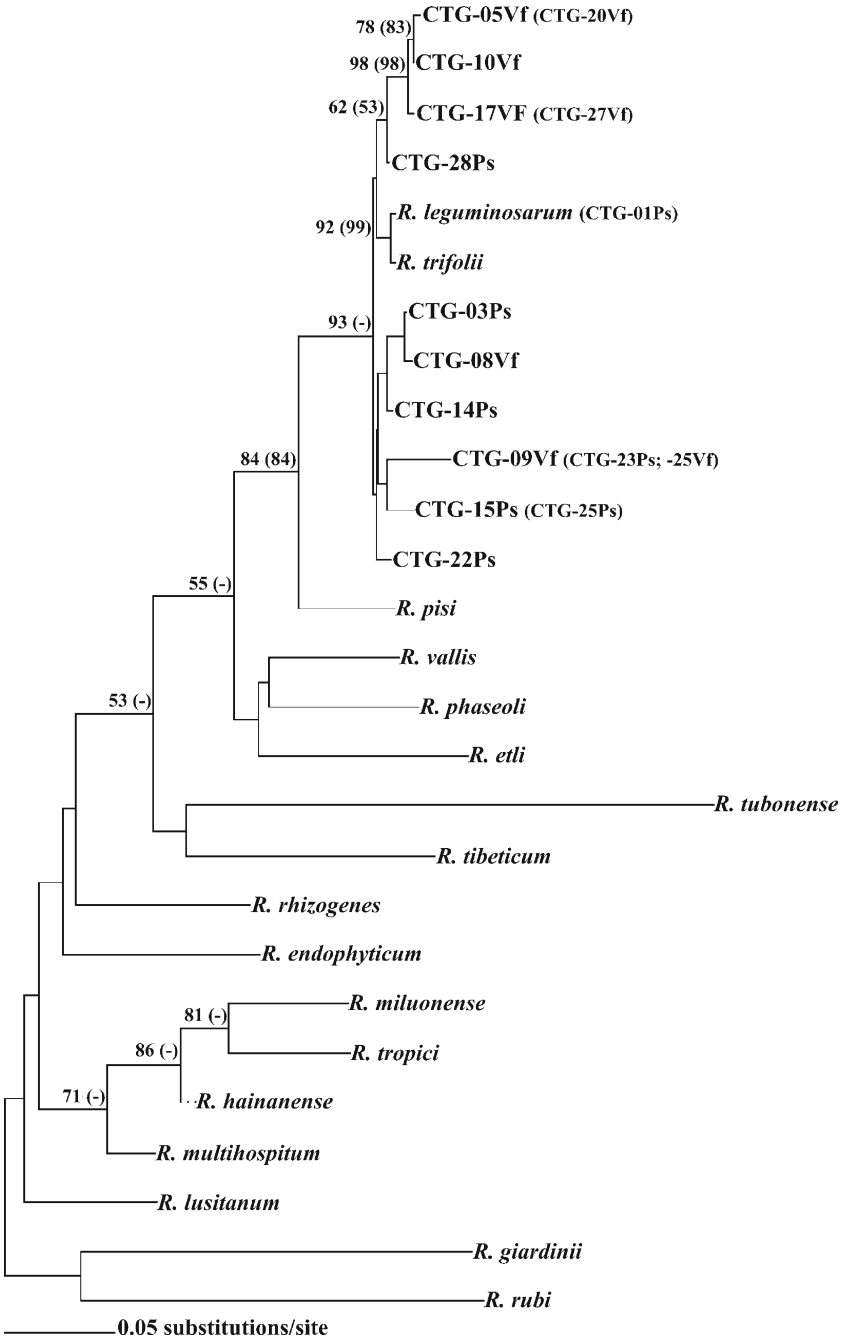

Fig. 3 NJ tree showing the phylogenetic relationships among *recA* haplotypes obtained in this study and type strains of some rhizobial species obtained from GenBank (below). Bootstrap values of the ML tree produced with the same substitution model (TIM2+I+G) are given in *parentheses*. Only the bootstrap values greater then 50 % are shown. Rhizobial type strains and their accession numbers for *recA* are as follow: *R. tropici*^{T} AJ294373; *R. rhizogenes*^{T} AJ294374; *R. etli*^{T} AJ294375; *R. leguminosarum*^{T} AJ294376 (Gaunt et al. 2001); *R. lusitanum*^{T} DQ431674 (Valverde et al. 2006); *R. rubi*^{T} AM182122; *R. giardinii*^{T} AM182123 (Martens et al. 2007); *R. pisi*^{T} EF113134; ATCC 14480 EF113135; *R. phaseoli*^{T} EF113136 (Santillana et al. 2008); *R. multihospitum*^{T} EF490029 (Han et al. 2008b); *R. tibeticum*^{T} EU288694 (Hou et al. 2009); *R. endophyticum*^{T} HM142767 (Lopez-Lopez et al. 2010); *R. miluonense*^{T} HM047131; *R. tubonense*^{T} EU288696 (Zhang et al. 2011); *R. vallis*^{T} GU211770 (Wang et al. 2011); *R. hainanense*^{T} HM047132 (Chang et al., unpublished); *R. indigoferae*^{T} EF027965 (unpublished)

sativum L.) that had the same *nodA* haplotype also fell in this group as a separate lineage. Each of other *Rlv* isolates in the first lineage, FB10061 (*V. faba* L. from Tunisia), BLR57 (*Lens culinaris* L. from Bangladesh), 201ZG (*P. sativum* L. from Crotia), USDA 2370 (*R. leguminosarum* type strain from *P.sativum* L. in USA) and VAS602 (*Vicia angustifolia* L. from South Korea), had a unique *nodA* haplotype. The nucleotide sequence similarity within this lineage varied between 95.7 and 99.7 %. Our two haplotypes, CTG-01Ps and CTG-28Ps, which were isolated from *P. sativum* L. root nodules, formed the third sublineage, with a 99.7 % nucleotide similarity. This sublineage appeared as sister to the first two sublineages with 96 % and 94 % bootstrap values in the NJ and ML trees, respectively. The fourth sublineage, which appeared as the most ancestral one in the *Rlv* lineage, was comprised of a single European originated haplotype, 301ZG. This haplotype appeared as sister to the other *Rlv nodA* haplotypes, with a 100 % bootstrap value in the ML.

Approximately 675 bp of the *nifH* gene were sequenced for the selected rhizobial isolates (Table 1). Phylogenetic analysis was conducted on 349 aligned nucleotides with 103 segregated sites. AIC and BIC tests suggested the TPM3uf+G (G:0.295) and TPM3+G (G: 0.374) substitution models, respectively (Fig. 5). We present the NJ and ML trees with

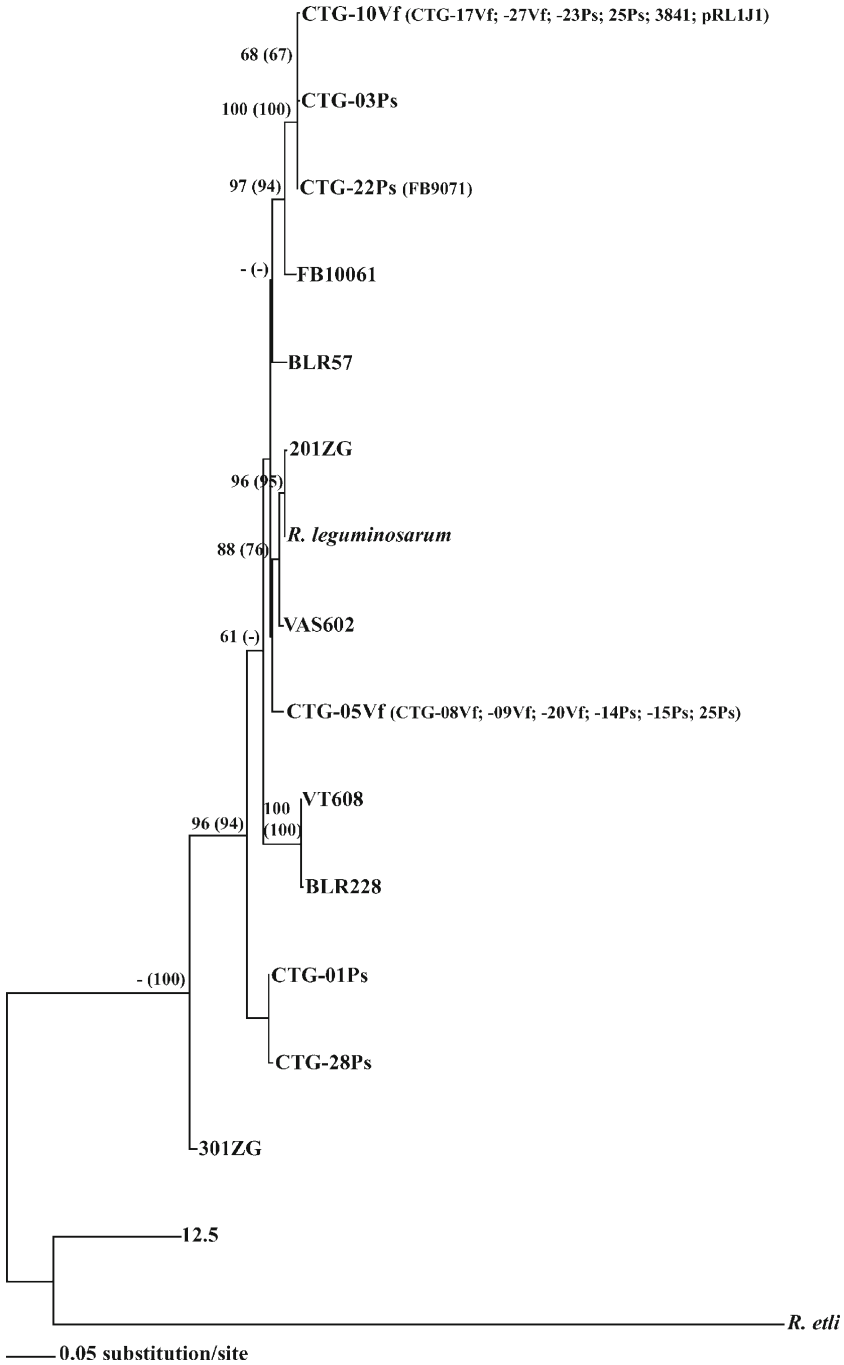

Fig. 4 NJ tree showing the phylogenetic relationships among *nodA* haplotypes obtained in this study and those from GenBank (below). Bootstrap values of the ML tree produced with the same substitution model (HKY+G) are given in *parentheses*. Only the bootstrap values greater then 50 % are shown. Rhizobial type strains and their accession numbers for *nodA* are as follow: *pRL1JI* Y00548 (Rossen et al. 1984); *R. etli*^{T} NC_004041 (Gonzalez et al. 2003); *pRL10* AM236084 (Young et al. 2006); *301ZG* DQ286867; *201ZG* DQ286900 (Zafran-Novak et al. 2010); *Strain 12.5* GQ374373 (Mazur et al. 2011); *BLR57* JN648986; *BLR228* JN648992 (Rashid et al. 2012); *VAS602* FJ650409; *VT608* FJ715818 (Kim et al. unpublished); *FB9071* JN558708; *FB10061* JN558709; *R. leguminosarum*^{T} JN558711 (Saidi et al. unpublished)

TPM3+G nucleotide substitution model because they showed higher bootstrap values. Both in the NJ and ML trees two main monophyletic groups appeared in the *Rlv nifH* haplotypes. The first monophyletic group contained 9 haplotypes from different continents, Europe (Belgium), Americas (USA), Asia (India, China) and Africa (Tunisia), and appeared as the most widespread one. The nucleotide sequence similarities within the lineage were between 96.5 and 98.2 %. Four of our isolates (CTG-01Ps, -03Ps; -22Ps and -28Ps) obtained from *P. sativum* L. also grouped in this lineage. Isolates CTG-28Ps had the same *nifH* haplotype as USDA 2370 (*R. leguminosarum* type strain from *P. sativum* L. from the USA) and R4532 (*Vicia cracca* L. from Belgium) and another isolate of ours, CTG-01Ps, had same haplotype as BIHB1192 (*P.sativum* L. from India). Furthermore, two of our isolates, CTG-03Ps and CTG-22Ps, shared a unique haplotype within the first lineage. On the other hand, the second lineage appeared relatively less disseminated and containined isolates from Turkey, North Africa (Tunisia and Morocco) and Europe (U.K.). Only two haplotypes fell within this lineage and had a 99.1 % nucleotide sequence similarity. The Turkish isolates CTG-10Vf, -17Vf,

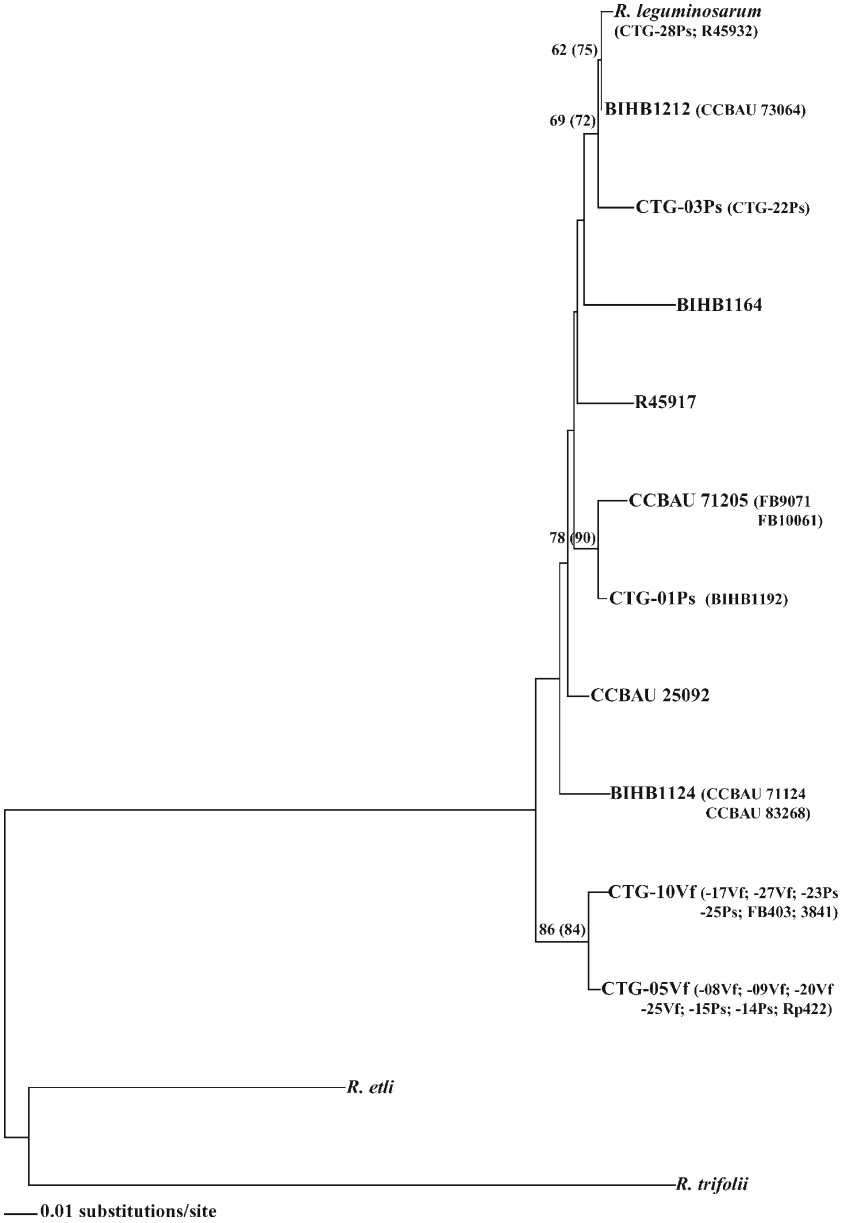

Fig. 5 NJ tree showing the phylogenetic relationships among *nifH* haplotypes obtained in this study and those from GenBank (below). Bootstrap values of the ML tree produced with the same substitution model (TPM3+G) are given in *parentheses*. Only the bootstrap values greater then 50 % are shown. Rhizobial type strains and their accession numbers for *nifH* are as follow: *USDA 2370* DQ450935 (Laranjo et al. 2008); *BIHB1212* JF759731; *BIHB1124* JF759708; *BIHB1164* JF759722; *BIHB1192* JF759727 (Rahi et al. 2012); *Isolate 384* K00490 (Scott et al. 1983); *R. etli*^{T} NC_004041 (Gonzalez et al. 2003); *Strain 3841* AM236084 (Young et al. 2006); *R45917* FR850696; *R45932* FR850699 (De Meyer and Willems 2011); *RP422* DQ413015 (Mouhsine et al., unpublished); *FB403* JN558693; *FB9071* JN558698; *FB10061* JN558699 (Saidi et al., unpublished); *CCBAU 71124* EU177595; *CCBAU 73064* EU177597; *CCBAU 71205* EU177596; *CCBAU 25092* EU177588 (Lei et al., unpublished); *CCBAU 83268* EU252583 (Han et al., unpublished)

-27Vf (from *V. faba* L.) and CTG-23Ps, -25Ps (from *P. sativum* L.) and isolates FB403 (*V. faba* L. from Tunisia) and 3841 (*P. sativum* L. from the U.K.) had the same *nifH* haplotype, whereas CTG-05Vf, -09Vf, -20Vf, -25Vf, -08Vf (from *V. faba* L.), CTG-14Ps, -15Ps (from *P. sativum* L.) and isolate RP422 (Morocco) had the second haplotype in this lineage. This lineage seemed relatively robust with 86 and 84 % bootstrap values in the NJ amd ML trees, respectively. The overall nucleotide sequence similarities among all *Rlv nifH* haplotypes were between 95.9 and 98.2 %.

To determine the *recA* haplotype relations within *Rlv*, network analysis was carried out over 414 aligned nucleotides with 68 segregated sites. In the *recA* network, three main lineages were revealed (Fig. 6). Most of our isolates from pea (CTG-03Ps, -14Ps, -15Ps, -22Ps, -25Ps) and also CTG-08Vf from faba bean grouped within lineage-I, together with faba bean *Rlv* isolates from Spain (USDA 2500, USDA 2502), China (CCBAU 81106, CCBAU 23131), Jordan (Nvf3) and India (BIHB 1160). The lineage-II appeared as a more homogenous one in terms of host and locality. All isolates in this lineage were from faba bean, except for CTG-28Ps and 3841 which were from pea. Moreover, no Asian haplotype occurred in this lineage, except for CCBAU 03058 from China. Isolates in this lineage were from Europe (3841, VF-39, USDA 2497, USDA 2503), the Middle East (J-1) or Americas (PEVF03, PEVF10, USDA 2489), besides Turkey (CTG-05Vf, -10Vf, -17Vf, -20Vf, -27Vf, -28Ps). Lineage-III was comprised of isolates from diverse localities. Most of the Asian haplotypes (CCBAU 81107, CCBAU 33204, CCBAU 03316, CCBAU 03321, CCBAU 43229, BIHB 1138, BIHB 1192, BIHB 1220)

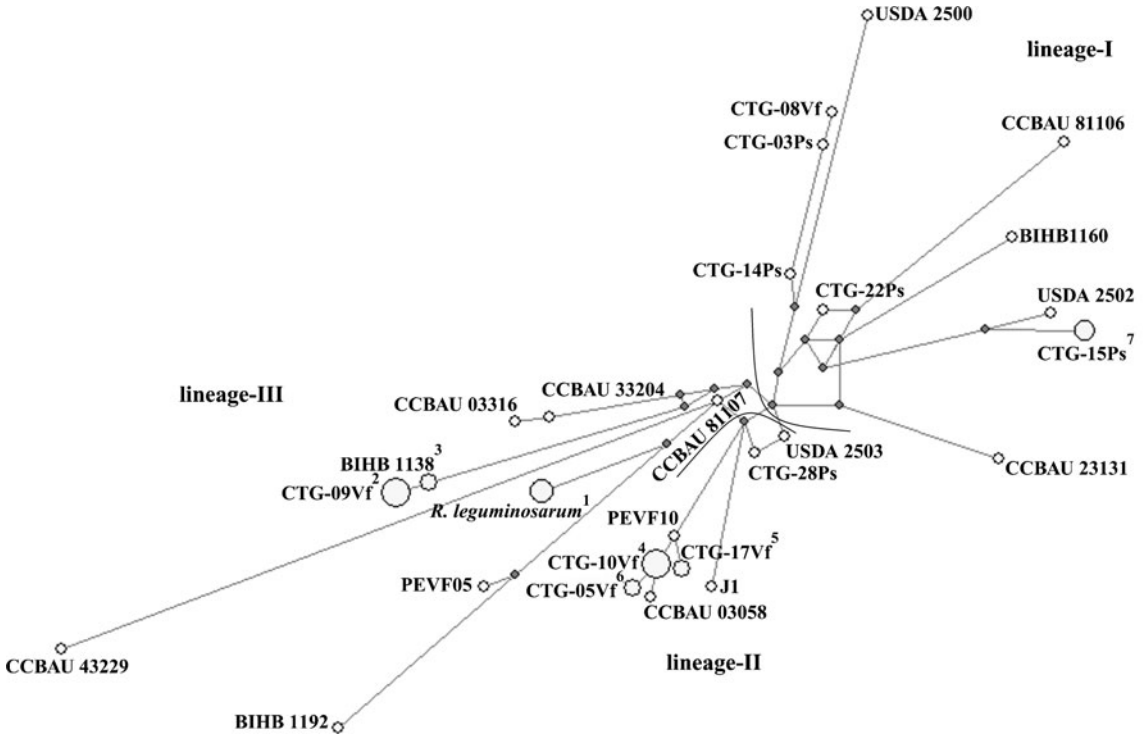

Fig. 6 Median-joining network of *recA* haplotypes obtained in this study together with the haplotypes retrieved from GenBank for *R. leguminosarum*. Haplotypes are denoted as *uncoloured circles* with a size proportional to haplotype frequency and the lengths of the branches are proportional to the number of mutational steps between haplotypes. Unsampled or missing nodes are indicated by *grey-coloured diamonds*. [1]CTG-01Ps, PEVF01, BIHB1220; [2]USDA2499, Nvf1, PEVF08, CTG-23Ps, CTG-25Vf; [3]CCBAU03321; [4]USDA2489, USDA2497, 3841, VF39, PEVF03; [5]CTG-27Vf; [6]CTG-20Vf; [7]Nvf3; CTG-25Ps. Rhizobial strains and their accession numbers for *recA* are as follow: *BIHB 1138* JF759773; *BIHB1160* JF759780; *BIHB1192* JF759787; *BIHB1220* JF759793 (Rahi et al. 2012); *CCBAU 03321* GQ323673; *CCBAU 23131* GQ323661; *J1* GQ323691; *USDA 2502* GQ323689; *Nvf3* GQ323694; *USDA 2503* GQ323690; *USDA 2489* GQ323684; *CCBAU 81106* GQ323671; *CCBAU 43229* GQ323665; *CCBAU 03058* GQ323681; *CCBAU 33204* GQ323659; *CCBAU 81107* GQ323675; *USDA 2499* GQ323687; *CCBAU 03316* GQ323678; *USDA 2500* GQ323688; *Nvf1* GQ323692; *USDA 2497* GQ323685 (Tian et al. 2010); *PEVF01* EF113122; *PEVF03* EF113124; *PEVF05* EF113125; *PEVF08* EF113126; *PEVF10* EF113128 (Santillana et al. 2008); *USDA2370* AJ294376 (Gaunt et al. 2001); *VF39* AY907362 (Vinuesa et al. 2005); *3841* AM236080 (Young et al. 2006)

appeared in this lineage, together with haplotypes from the Middle East (Nvf1), Europe (USDA 2499), Americas (PEVF01, PEVF05, PEVF08) and Turkey (CTG-01Ps, -23Ps, -09Vf, -25Vf).

Separately, no appropriate-sized PCR product (approx. 1100 bp) was detected in the 60 rhizobial isolates tested for the presence of the *nodX* gene.

Discussion

In this study, we analysed a rhizobial collection consisting of 60 isolates obtained from *Vicia faba* L. (n = 30) and *Pisum sativum* L. (n = 30) root nodules collected from four different provinces in the central Black Sea region of Turkey by using 16S rDNA RFLP analysis and 16S rDNA, *recA*, *nodA* and *nifH* nucleotide sequence phylogenies.

All rhizobial isolates obtained in this study showed the same 16S rDNA RFLP pattern (Fig. 1), indicating that they belong to the same species. Isolates (n = 16) selected as representatives for nucleotide sequencing (Table 1) showed a close relationship with the *R. leguminosarum* isolates USDA 2370 (Type strain) and ATCC 14480 (bv. *trifolii*) from the 16S rDNA (Fig. 2) and *recA* (Fig. 3) phylogenetic trees. The nucleotide sequence similarities between our isolates and the *R. leguminosarum* type strain (USDA 2370) for the 16S rDNA (99.5–99.9 %) and *recA* (96.7–99.4 %) also supported these findings, suggesting that *R. leguminosarum* is the dominant symbiont of faba bean and pea in the north part of Turkey, as previously reported from *Phaseolus vulgaris* L. in the same locality (Gurkanli et al. 2012).

Our *recA* network analysis revealed three main lineages (Fig. 6) within *Rlv* isolates originating from different countries. Lineage-I was comprised of isolates from Asia, Europe and the Middle East, but had no isolates from the Americas. Turkish isolates in this lineage showed close relationships with Spanish and Jordanian isolates, rather than with Asian isolates. On the other hand, lineage-II had isolates only from Europe, the

Americas and the Middle East (the only exception was CCBAU 03058). Lineage-III appeared to be the more global one, consisting of *Rlv* isolates from Asia, Europe, Americas and the Middle East (Turkey and Jordan). As in lineage-I, the Turkish *Rlv* isolates in lineage-III also grouped with Spanish, Jordanian and Peruvian isolates. These findings clearly suggest a common evolutionary history for Turkish, European and Jordanian *Rlv* isolates, as well as those from the Americas. The genetic diversity of Turkish, European and Jordanian *Rlv* isolates, and also those from the Americas, distributed across all three lineages, was higher than that of the Asian isolates predominantly found in lineages -I and -III, with one exception. Although it has been suggested that the host plants *V. faba* L. and *P. sativum* L. probably originated from the Middle East or South-west Asia, both our findings and those of Álvarez-Martínez et al. (2009) may suggest that their microsymbiont (*Rlv*) originated from Europe or the Middle East.

Because of the genetic locus $sym2^A$ in their genomes, most wild-type "primitive" pea cultivars from Afganistan are resistant to nodulation by *Rlv* isolates originating from Western Europe and North America (Holl 1975; Lie 1978; Kozik et al. 1995). On the other hand, some isolates are able to nodulate these plants due to the presence of an extra nodulation gene, *nodX*, coding for an *O*-acetyl transferase on their sym plasmid, that was first identified from a Turkish isolate TOM (Davis et al. 1988). *Rhizobium leguminosarum* bv. *viciae* isolates that can nodulate Afghanistan-type pea have been reported from several countries, including Denmark (Jensen et al. 1986), the former Soviet Union (Chetkova and Tikhonovich 1986), and China, India, Morocco and Yugoslavia (Ma and Iyer 1990). In addition to these countries, most of the Turkish *Rlv* isolates were reported to be inducers of nodules on Afghanistan-type pea (Lie 1978). However, our findings conflict with that report because we could not amplify *nodX* from any of our isolates. This result may suggest multiple origins for Turkish *Rlv* isolates. Given the close relationship among the West European, Americas, Jordanian and Turkish *Rlv* isolates in the *recA* network, and the absence of the *nodX* gene in Turkish isolates, the Turkish *Rlv* isolates from the central Black Sea region and those from Western Europe and the Americas might have had a common evolutionary history.

It is also worth pointing out that most of our isolates obtained from faba bean root nodules (CTG-05Vf, CTG-10Vf, CTG-17Vf, CTG-20Vf, CTG-27Vf) grouped in the same *R. leguminosarum*-related lineage where pea isolates (except CTG-28Ps) formed a second lineage related to *R. leguminosarum* both in 16S rDNA and *recA* trees. Several studies have suggested that *V. faba* has been nodulated by a special group of *Rlv* isolates that are somehow different from the other *Rlv* isolates (Hynes and O'Connell 1990; Van Berkum et al. 1995). Our findings may provide molecular evidence supporting that hypothesis. However, our results for the symbiotic elements *nodA* and *nifH* are not consistent with 16S rDNA and *recA* phylogenies because the same *nodA* and *nifH* haplotypes were shared both by faba bean and pea rhizobial isolates grouped in different *Rlv*-related lineages in both the 16S rDNA and *recA* trees. This may indicate that the specialised relationship between faba bean and *Rlv* is not attributable to the nodulation factors secreted by the bacteria but may be due to a step earlier in the nodulation process. There is a clear need for more data to test this hypothesis.

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