RESEARCH LETTER

Stable low molecular weight RNA profiling showed variations within *Sinorhizobium meliloti* and *Sinorhizobium medicae* nodulating different legumes from the alfalfa cross-inoculation group

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Abstract

Four different low molecular weight (LMW) RNA profiles, designated I–IV, among 179 isolates from *Medicago*, *Melilotus* and *Trigonella* species growing in a field site in Northern Spain were identified. From sequence analysis of the 16S rRNA, *atpD* and *recA* genes as well as DNA–DNA hybridization analysis with representatives of each LMW RNA profile it was evident that isolates with LMW RNA profiles I and II belonged to *Sinorhizobium meliloti* and those displaying profiles III and IV to *Sinorhizobium medicae*. Therefore, two distinct LMW RNA electrophoretic mobility profiles were found within each of these two species. Collectively, LMW RNA profiles I and II (identified as *S. meliloti*) were predominant in *Melilotus alba, Melilotus officinalis* and *Medicago sativa*. Profiles III and IV (identified as *S. medicae*) were predominant in *Melilotus parviflora*, *Medicago sphaerocarpa*, *Medicago lupulina* and *Trigonella foenum-graecum*. All the four LMW RNA profiles were identified among isolates from *Trigonella monspelliaca* nodules. These results revealed a different specificity by the hosts of the alfalfa cross-inoculation group towards the two bacterial species found in this study.

Introduction

The diversity of rhizobia nodulating *Medicago* species originating from Europe (Paffetti *et al.*, 1996, 1998; Carelli *et al.*, 2000; Biondi *et al.*, 2003; Bradic *et al.*, 2003), Africa (Jebara *et al.*, 2001; Badri *et al.*, 2003; Zribi *et al.*, 2004), Asia (Roumiantseva *et al.*, 2002) and America (del Papa *et al.*, 1999; Silva *et al.*, 2007) is extensive. A broad collection that originated from various locations throughout the world was examined (Eardly *et al.*, 1990) and subsequently, van Berkum *et al.* (2006) reported that this collection consisted of 91 different chromosomal sequence types (STs) based on results from multi-locus sequence typing (MLST). Currently, three bacterial species *Sinorhizobium meliloti* (de Lajudie *et al.*, 1994), *Sinorhizobium medicae* (Rome *et al.*, 1996) and *Rhizobium mongolense* (van Berkum *et al.*, 1998), that nodulate different *Medicago* species have been proposed.

The symbiosis between *Medicago sativa* (alfalfa) and *S. meliloti* is probably the most studied (Gage, 2004) and the complete genome sequence of the alfalfa isolate strain 1021 has been published (Capela *et al.*, 2001). Apart from the presence of diversity among a few rhizobial isolates of *Trigonella foenum-graecum* and *Melilotus officinalis* in a limited study of medicinal legumes in the sub-Himalayan region of Uttaranchal, India (Pandey *et al.*, 2004) no additional reports about rhizobia of *Melilotus* and *Trigonella* are available even though they are within the *Medicago* cross-inoculation group. Information about rhizobia that form symbioses with these legume genera is necessary because many are important cover crops in programs to preserve the environment and are pioneer plants in ecosystems that are degraded by fire and by pollutants. Under these circumstances the nitrogen-fixing symbioses of these legumes promotes soil enrichment essential for the establishment of other nonleguminous plants.

Both *Trigonella* and *Melilotus* are part of the natural vegetation in Northern Spain. Because no information about their symbioses is available, the objective of this work was to explore the potential for variability of rhizobia that infect species of these two legume genera and to compare results

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obtained with isolates from several species of *Medicago* growing at the same field sites. The approach used was to determine variation in electrophoretic mobilities of low molecular weight (LMW) RNA because many determinations are possible in short periods of time (Jarabo-Lorenzo *et al.*, 2000; Palomo *et al.*, 2001) and results have been reported to correspond with 16S rRNA gene sequences (Velázquez *et al.*, 2001). Sequence analysis of the *rrs, recA* and *atpD* loci as well as DNA-DNA hybridizations with several representatives were used in support of the data because two unusual LMW RNA profiles were discovered among some of the isolates.

Materials and methods

Isolation from plant nodules

Isolations were made from root nodules of *M. sativa*, *Medicago lupulina*, *Medicago sphaerocarpa*, *Melilotus albus*, *M. officinalis*, *Melilotus parviflorus*, *Trigonella monspelliaca* and *T. foenum-graecum* (Table 1) according to Vincent (1970) using yeast mannitol agar (YMA). Seeds of these legumes were surface sterilized and sown in a soil from Riego de la Vega (Northern Spain). The legumes *T. foenum-graecum* and *M. sativa* have never been cultivated in this soil, but all the legume species that were tested are part of the natural vegetation in this location of Spain. Plants were grown in a greenhouse without supplemental lighting for 30 days. At harvest nodules were randomly selected from 10 plants within each legume species used as trap host.

LMW RNA analysis

LMW RNA extraction using the phenol/chloroform method was as described by Höfle (1988). The mixtures of LMW RNA from each extraction were separated according to molecular size by staircase electrophoresis (Cruz-Sánchez

Table 1. Host of origin, the number of isolates and the percentage of isolates with each LMW RNA profile

Host	**Total**	LMW RNA genotype I	LMW RNA genotype II	LMW RNA genotype III	LMW RNA genotype IV
Melilotus alba		RMA01, RMA02, RMA03, RMA04, RMA05, RMA06, RMA07, RMA08, RMA09, RMA12, RMA13, RMA14, RMA15, RMA16, RMA17, RMA21, RMA24, RMA25, RMA26, RMA28, RMA29	RMA20, RMA30, RMA31, RMA32, RMA33		
Subotal and percentages	**26**	**21 (77%)**	**5 (19%)**	**0**	**0**
Melilotus officinalis		RMOF02, RMOF03, RMOF04, RMOF05, RMOF06, RMOF07 RMOF08, RMOF09, RMOF11, RMOF12, RMOF13, RMOF14 RMOF15, RMOF18, RMOF20	RMOF01		
Subtotal and percentages	**16**	**15 (94%)**	**1 (6%)**	**0**	**0**
Melilotus parviflora				RMP02, RMP03, RMP04, RMP05, RMP06, RMP07, RMP08 RMP10, RMP11, RMP12, RMP13, RMP14, RMP18, RMP19 RMP20, RMP21, RMP22, RMP23, RMP24, RMP25, RMP27 RMP29, RMP30	RMP01
Subtotal and percentages	**24**	**0**	**0**	**23 (96%)**	**1 (4%)**

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Table 1. Continued.

Host	**Total**	LMW RNA genotype I	LMW RNA genotype II	LMW RNA genotype III	LMW RNA genotype IV
Trigonella monspelliaca		RTM03, RTM10, RTM12, RTM14, RTM16, RTM18, RTM21, RTM23, RTM24	RTM02, RTM06, RTM08, RTM11, RTM15, RTM17	RTM01, RTM05, RTM07, RTM09, RTM19, RTM22	RTM04
Subtotal and percentages	**22**	**9 (37%)**	**6 (27%)**	**6 (27%)**	**1 (4%)**
Trigonella foenum-graecum			RTF15	RTF01, RTF02, RTF03, RTF07, RTF08, RTF09, RTF13 RTF14 RTF17, RTF18, RTF19, RTF20, RTF21, RTF22 RTF24, RTF25	RTF04, RTF06, RTF11, RTF12
Subtotal and percentages	**21**	**0**	**1 (5%)**	**16 (76%)**	**4 (19%)**
Medicago sphaerocarpa		RMS10		RMS04, RMS06, RMS07, RMS08, RMS09, RMS11, RMS12 RMS15, RMS16, RMS18, RMS19, RMS20, RMS22, RMS23, RMS26 RMS28, RMS29	RMS01, RMS02, RMS21, RMS24
Subtotal and percentages	**22**	**1 (4%)**	**0**	**17 (72%)**	**4 (18%)**
Medicago lupulina				RML01, RML02, RML03, RML05, RML06, RML09, RML11 RML13, RML15, RML16, RML17, RML20, RML21, RML24 RML27, RML28, RML29, RML30	RML04, RML14, RML22, RML23
Subtotal and percentages	**22**	**0**	**0**	**18 (82%)**	**4 (18%)**
Medicago sativa		RMSA01, RMSA03, RMSA05, RMSA06, RMSA07, RMSA08 RMSA09, RMSA10, RMSA11, RMSA12, RMSA17, RMSA19 RMSA22, RMSA24, RMSA28, RMSA30, RMSA31, RMSA33 RMSA37, RMSA43, RMSA46, RMSA51	RMSA04, RMSA36, RMSA42	RMSA49	
Subtotal and percentages	**26**	**22 (85%)**	**3 (11%)**	**1 (4%)**	**0**
Total and percentages	**179**	**67 (37%)**	**15 (8%)**	**80 (45%)**	**15 (8%)**

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et al., 1997) using 400 mm × 360 mm × 0.4 mm gels in a vertical slab unit (Poker Face SE 1500 Sequencer, Hoeffer Scientific Instruments, San Francisco, CA). The separating gel contained 14% acrylamide/Bis (acrylamide: *N*,*N*-methylene bisacrylamide 29 : 1 (w/w), 7 M urea in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.5). The running buffer was TBE (× 1.2) that was cycled at a flow rate of 300 mL min^{-1} using a peristaltic pump (MasterFlex, Cole Parmer Instruments, Chicago, IL). Before loading the samples the gel was stabilized with a pre-electrophoretic run for 30 min at 100 V and 50 °C. Samples for analysis containing 3 μg of RNA measured spectrophotometrically were added to 5 μg of loading solution (300 mg mL^{-1} of sucrose, 460 mg mL^{-1} of urea, 10 μL mL^{-1} 20% sodium dodecylsulfate, 1 mg mL^{-1} xylene cyanol) and were subsequently heated at 70 °C for 10 min before loading. References used in the electrophoretic runs were obtained from Boehringer Manheim (Manheim, Germany) and Sigma (St Louis, MO) and included: 5S rRNA gene from *Escherichia coli* MRE 600, tRNA specific for tyrosine from *E. coli* and tRNA specific for valine from *E. coli*. The gels were silver-stained according to the method described by Haas *et al.* (1994) subsequent to electrophoresis.

Determination of *rrs*, *recA* and *atpD* gene sequences and analysis of the sequence data

PCR amplification and sequence analysis of the 16S rRNA genes was according to van Berkum *et al.* (1996) and that of *recA* and *atpD* genes was as described by Gaunt *et al.* (2001). The sequences obtained were compared with those from GenBank using the BLASTN program (Altschul *et al.*, 1990) and were aligned using the CLUSTAL W software (Thompson *et al.*, 1997). The distances among the sequences were determined according to Kimura's two-parameter method (Kimura, 1980) and phylogenetic trees were generated using the neighbour-joining method (Saitou & Nei, 1987) Bootstrap analysis was based on 1000 resamplings. The MEGA 2.1.0 package (Kumar *et al.*, 2001) was used for all analyses.

Randomly amplified polymorphic DNA (RAPD) patterns and DNA–DNA hybridization analysis

RAPD patterns was used to compare the type strains of *S. meliloti* and *S. medicae* from different culture collections used in different experiments of this study. To obtain them we used the primer M13 (5′-GAGGGTGGCGGTTCT-3′) in the conditions reported by Rivas *et al.* (2006).

DNA–DNA hybridization analysis was according to Ezaki *et al.* (1989) following the recommendations of Willems *et al.* (2001). The analysis was done with representative strains from each of the four LMW RNA groups and also included the type strains for *S. meliloti* LMG 6133^T and *S. medicae* USDA 1037^T.

Results and discussion

LMW RNA analysis

Variation in electrophoretic mobilities of cellular LMW RNA produce distinct fingerprint profiles that previously have been shown to be useful molecular markers in distinguishing different genera and species of rhizobia (Höfle, 1988; Velázquez *et al.*, 1998, 2001). From the LMW profiles obtained with the 179 isolates in this study it was concluded that all exhibited 5S rRNA gene zones that were typical of the genus *Sinorhizobium* and not *Rhizobium* (Velázquez *et al.*, 1998) when compared with the type strains for *S. meliloti* (lane 1, Fig. 1), *S. medicae* (lane 12, Fig. 1) and *R. mongolense* (lane 24, Fig. 1). The isolates were placed into four distinct groups based upon the LMW RNA profile they exhibited (Fig. 1, Table 1). The LMW RNA profile I (lanes 1–6, Fig. 1) corresponded with the type strain for *S. meliloti* USDA 1002^T (LMG 6133^T) and the profile III (lanes 12–18, Fig. 1) was identified with the type strain for *S. medicae* USDA 1037^T. Besides these two profiles that clearly identified *S. meliloti* and *S. medicae* among the isolates, two additional profiles were observed, profiles II (lanes 7–11, Fig. 1) and IV (lanes 19–23), respectively. Because these legume species normally are nodulated by *S. meliloti* (profile I) and *S. medicae* (profile III) and we found two additional LMW RNA profiles among the strains isolated in this study we performed several additional techniques to confirm their identification.

Analysis of 16S rRNA (*rrs*) gene

Representative isolates for each of the four LMW RNA profiles were randomly chosen and by sequence analysis of the 16S rRNA genes were placed within the genus *Sinorhizobium* (Fig. 2). The sequences of RMOF18 and RTM17, representatives of LMW RNA profiles I and II, respectively, had 16S rRNA gene sequences that were 100%, and 99.9% similar to the sequence of *S. meliloti* LMG 6133^T. The 16S rRNA gene sequences of RMP05 and RMP01 that represented LMW RNA profiles III and IV, respectively, were identical to the sequence of *S. medicae* USDA 1037^T. Therefore, isolates with LMW RNA profiles I and II are closely related to each other and would support the suggestion that members of *S. meliloti* have two different LMW RNA profiles. Similarly, isolates with LMW RNA profiles III and IV are closely related to each other and would support the suggestion that members of *S. medicae* have two different LMW RNA profiles. However, resolution of 16S rRNA gene sequence divergence with placement of isolates at the species level is poor (Valverde *et al.*, 2006) and species level interpretations may be inaccurate because of the reported evidence that the 16S rRNA genes in rhizobia have a history of genetic recombination (van Berkum *et al.*, 2003).

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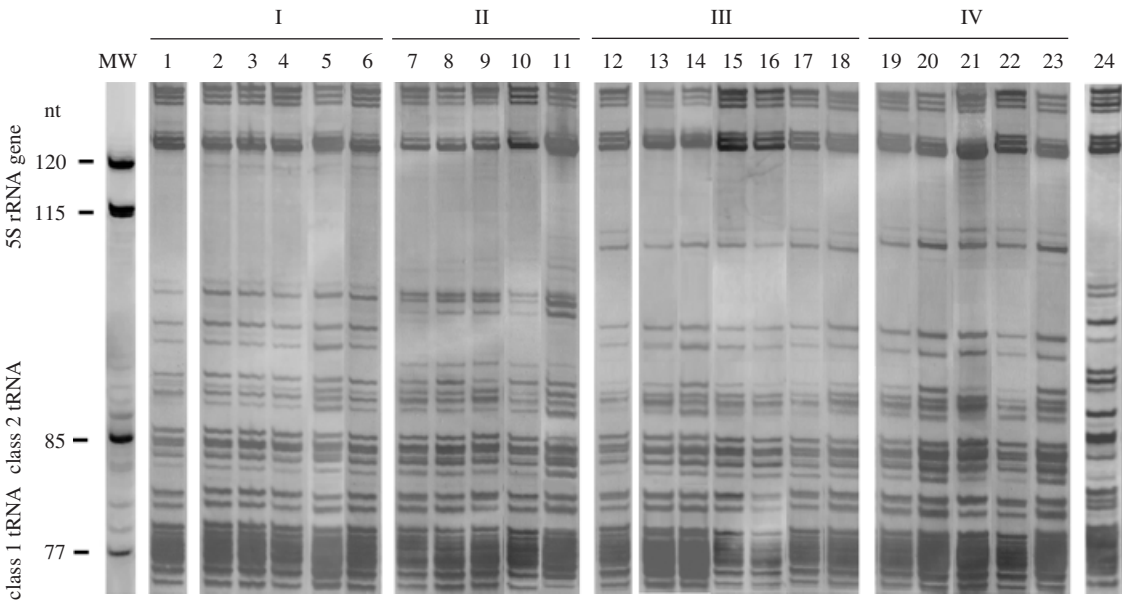

Fig. 1. LMW RNA profile I: *Sinorhizobium meliloti* USDA 1002^T (lane 1), RMOF18 (lane 2), RMA01 (lane 3), RMSA01 (lane 4), RMS10 (lane 5) and RTM03 (lane 6). LMW RNA profile II: RTM17 (lane 7), RTF15 (lane 8), RMOF01 (lane 9) RMA32 (lane 10) and RMSA04 (lane 11) LMW RNA profile III: *Sinorhizobium medicae* USDA 1037^T (lane 12), RMP05 (lane 13), RMS04 (lane 14), RML01 (lane 15), RMSA49 (lane 16), RTF01 (lane 17) and RTM05 (lane 18). LMW RNA profile IV: RMP01 (lane 19), RMS01 (lane 20), RML04 (lane 21), RTM04 (lane 22) and RTF06 (lane 23). LMW RNA profile of *Rhizobium mongolense* USDA 1844^T (lane 24).

Consequently, further evidence in support of the placement of the isolates with LMW profile II within *S. meliloti* and for LMW profile IV within *S. medicae* was obtained from sequence analysis of *recA* and *atpD*.

Analysis of *recA* and *atpD* genes

The *rec*A alleles (Fig. 3a) of isolates RMOF08 and RTM17 that represented isolates with LMW RNA profiles I and II, respectively, were 99% similar to the allele of *S. meliloti* USDA 1002^T (= LMG 6133)^T. Isolates RMP05 and RMP01 that represented isolates with LMW RNA profiles III and IV, respectively, had *recA* alleles (Fig. 3a) that were identical and 99.6% similar with the allele of *S. medicae* USDA 1037^T (LMG 19920^T). The *atpD* alleles (Fig. 3b) of isolates RMOF08 and RTM17 were 99% similar to each other and were 99% and 98.2% similar to the allele of *S. meliloti* USDA 1002^T (= LMG 6133)^T. The *atpD* alleles of RMP01, RMP05 and *S. medicae* USDA 1037^T were identical (Fig. 3b). Therefore, the results of the sequence analysis of the partial *rec*A and *atp*D loci would support a conclusion that rhizobia with LMW RNA profiles II and IV should be classified as *S. meliloti* and *S. medicae*, respectively.

DNA–DNA hybridization analysis

DNA–DNA hybridization analysis was done using reference strains for the species *S. meliloti* and *S. medicae* that were from a different source than those that were used in the other analyses. Consequently, a RAPD analysis was done to ensure that these reference cultures and those used in the other analyses were interchangeable. The RAPD profiles obtained with the *S. meliloti* reference cultures ATCC 9930^T and LMG 6133^T were identical. Similarly, the RAPD results obtained with *S. medicae* USDA 1037^T and LMG 19920^T also were indistinguishable (data not shown). Subsequently, the strains LMG 6133^T and LMG 19920^T were used in the DNA–DNA hybridization analysis. The hybridization values (Table 2) clearly demonstrate that LMW RNA types I and II represent *S. meliloti*, whereas LMW RNA types III and IV represent *S. medicae*. Within each of these species, the LMW RNA types cannot be distinguished through the DNA-DNA hybridization data when the recommendation of a threshold value of 70% DNA–DNA similarity for definition of species is considered (Wayne *et al.*, 1987). Nevertheless, as expected taking into account the differences found in the tRNA profiles, the values obtained between the isolates displaying LMW RNA profiles I and II were lower than those found between those displaying LMW RNA profiles III and IV.

Plant-host distribution of the isolates

Among the collection of 179 isolates, those with LMW RNA profiles I and III were the most frequently encountered (37% and 45% of the isolates, respectively), while the detection of LMW RNA profiles II and IV was far less frequent, (8% of the isolates for each profile, respectively). Most of the isolates with LMW RNA profile I originated from *M. sativa*,

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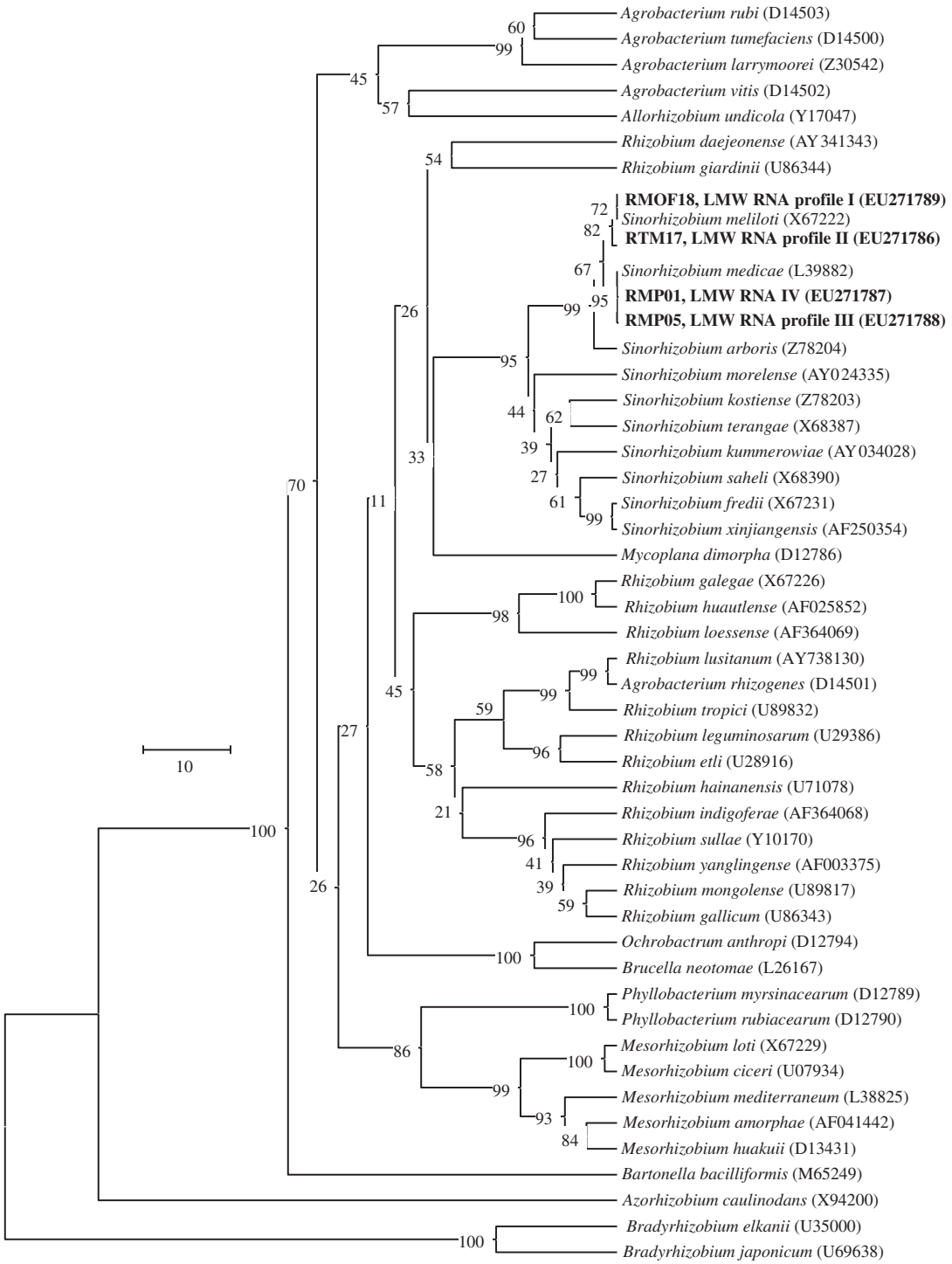

Fig. 2. Neighbour-joining tree constructed from sequence divergence of the 16S rRNA genes expressed in Jukes–Cantor distances. Bootstrap values were obtained with 500 permutations of the data set. Scale bar, 1 nt substitution per 100 nt.

M. officinalis and *M. alba* nodules, while those displaying LMW RNA profile III were obtained from *M. sphaerocarpa*, *M. lupulina*, *M. parviflora* and *T. foenum-graecum* nodules. The distribution of the LMW RNA profiles among the isolates obtained from the other legume hosts was more restricted (Table 1). Nodules of *T. foenum-graecum*, *M. lupulina*, *M. sphaerocarpa* and *M. parviflora* predominantly yielded rhizobia displaying LMW RNA profiles III

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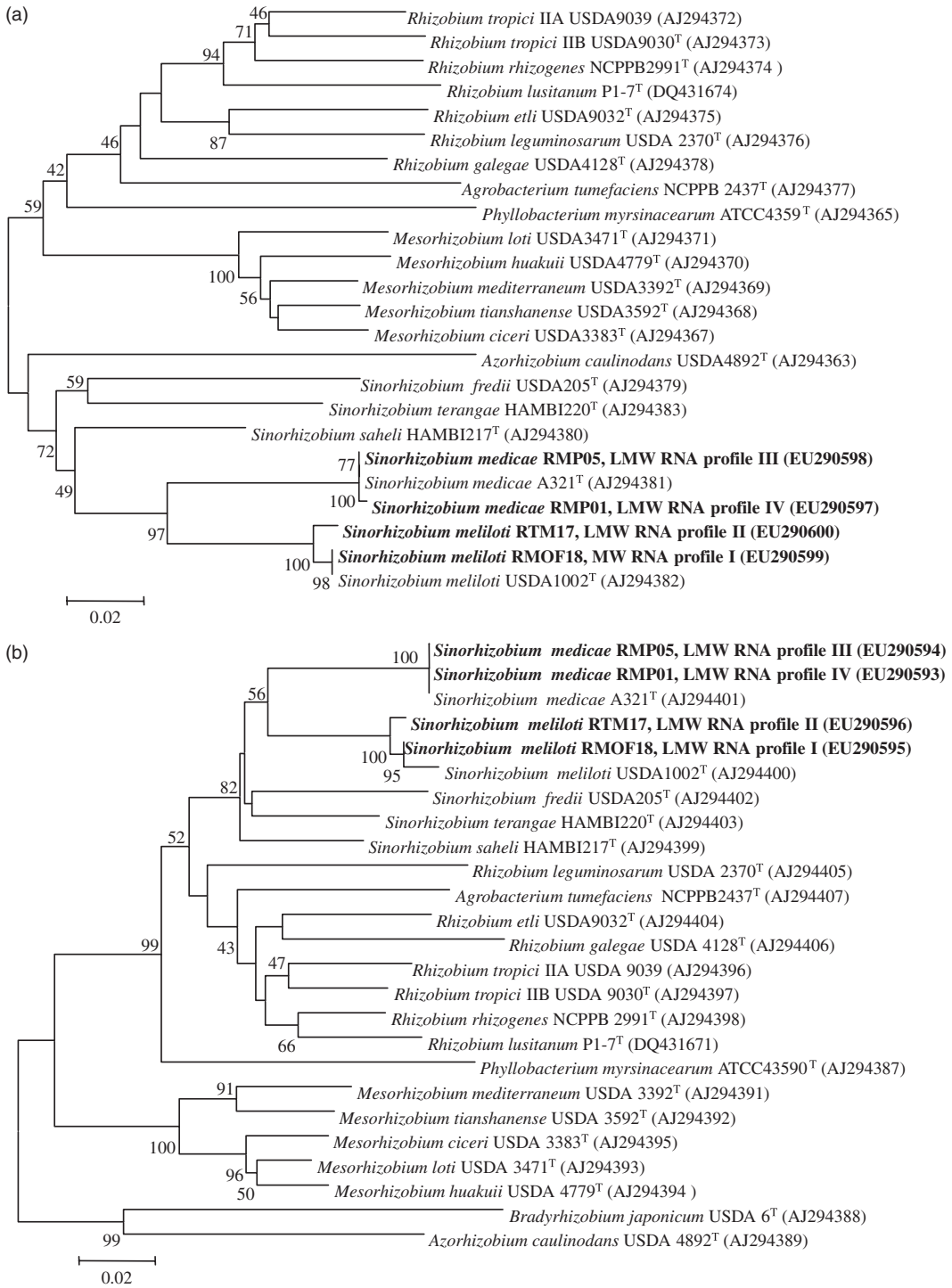

Fig. 3. Neighbour-joining tree constructed from divergence of partial *recA* (a) and *atpD* (b) sequences (520 nt) showing placement of isolate representatives for each of the LMW RNA profiles. Bootstrap values calculated for 1000 permutations are indicated. Scale bar, 2 nt substitution per 100 nt.

and IV that were identified as *S. medicae*. In contrast, rhizobia isolated from *M. officinalis*, *M. alba* and *M. sativa* nodules, with the only exception of strain RMSA49 (LMW RNA profile III), had LMW RNA profiles I and II that were identified as *S. meliloti*. These results showed some degree of specificity for nodulation of the two *Sinorhizobium* species towards the different hosts analysed with *S. meliloti* nodulating preferentially *M. alba*, *M. officinalis* and *M. sativa*, and

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Table 2. DNA–DNA hybridization values

Strain	RTM17 (genotype I)	RMOF18 (genotype II)	*S. meliloti* LMG 6133^T	RMP01 (genotype IV)	RMP05 (genotype III)	*S. medicae* LMG 19920^T
RTM17 (LMW RNA type II)	100					
RMOF18 (LMW RNA type I)	90	100				
S. meliloti LMG 6133^T	83	93	100			
RMP05 (LMW RNA type III)	54	48	45	100		
RMP01 (LMW RNA type IV)	53	48		93	100	
S. medicae LMG 19920^T	55	47		98	100	100

S. medicae mostly *M. parviflora*, *T. foenum-graecum*, *M. sphaerocarpa* and *M. lupulina*. Only the rhizobia recovered from nodules of *T. monspelliaca* displaying the four LMW RNA profiles and thus specificity for nodulation would not be indicated in this case.

Collectively, the frequency by which *S. medicae* was isolated was slightly higher (53%) than *S. meliloti* (47%). Similarly, a predominance of *S. medicae* in *Prosopis alba* nodules grown in Northern Spain has been recently reported (Iglesias *et al.*, 2007). However, the observation of a predominance of *S. medicae* in the nodules of *M. lupulina* and *T. foenum-graecum* grown in Spanish soil contrasts the report of Silva *et al.* (2007) with *M. lupulina* in Mexican soil and Roumiantseva *et al.* (2002) with *Trigonella* in Central Asian soil. The predominance of *S. meliloti* in nodules of *M. sativa* agrees with previous reports (Rome *et al.*, 1996; Carelli *et al.*, 2000; Roumiantseva *et al.*, 2002; van Berkum *et al.*, 2006). Therefore, the results of the present work add new information about the specificity patterns of some local hosts from the alfalfa cross-inoculation group.

The intraspecific genetic variability detected in *S. medicae* so far has been lower than in *S. meliloti* (Bailly *et al.*, 2006; van Berkum *et al.*, 2006; Silva *et al.*, 2007). However, in this work differences in tRNA profiles were found within these two rhizobial species that form symbioses with legumes that are part of the alfalfa cross-inoculation group. Therefore, LMW RNA analysis is a new way of exploring intraspecific diversity in rhizobia in which this procedure was mainly used to assess genus or species level variations (Velázquez *et al.*, 1998).

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