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ORIGINAL PAPER

Diversity and nodulation effectiveness of rhizobia and mycorrhizal presence in climbing dry beans grown in Prespa lakes plain, Greece

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Abstract

The Prespa lakes plain is an isolated area where about 1000 ha are seeded to *Phaseolus vulgaris* L. and *Phaseolus coccineus* L. Nodulation, arbuscular mycorrhizal fungal (AMF) presence and the genetic diversity of rhizobia were evaluated by 16S-ITS-23S-RFLP patterns and by sequencing. The bean rhizobial population in the region was diverse, despite its geographic isolation. No biogeographic relationships were detected, apart from a *Rhizobium tropici*-related strain that originated from an acidic soil. No clear pattern was detected in clustering with bean species and all isolates formed nodules with both bean species. Most strains were related to *Rhizobium leguminosarum* and a number of isolates were falling outside the already characterized species of genus *Rhizobium*. Application of heavy fertilization has resulted in high soil N and P levels, which most likely reduced nodulation and AMF spore presence. However, considerable AMF root length colonization was found in most of the fields.

Keywords Arbuscular mycorrhizae · *Phaseolus vulgaris* · *Phaseolus coccineus* · Symbiotic nitrogen fixation

Introduction

Rhizobia and the endomycorrhizal fungi of the phylum Glomeromycota form mutually beneficial relationships with legumes and most of the land plants, respectively. Tripartite symbiosis, plant-rhizobia-fungi, beneficial for all three, also takes place in legumes, with the plant providing photosynthetic carbon, rhizobia nitrogen (N) fixed from the atmosphere and the arbuscular mycorrhizal fungi (AMF) mostly phosphorus (P) and other immobile soil nutrients (Smith and Read 1997). In legumes, N_2-fixation leads to greater P need that may be met by AMF, and consequently, the AM symbiosis may increase the amount of N_2 fixed by the legume-rhizobium symbiosis (Redecker et al. 1997). Plant varieties may differ in their response to particulate AMF depending on soil properties (Hayman 1986). Furthermore, the mycorrhizal symbiosis may limit crop losses to pathogens (Dar et al. 1997). In low input agricultural systems, both symbiotic relationships may prove promising after isolation, evaluation and application of effective strains, and development of high-quality inocula.

Bean (*Phaseolus* L.) is an important legume crop which is known for low nodulation ability and symbiotic N_2-fixation that may not meet plant N demands (Havlin et al. 2014; Graham and Ranalli 1997). As a result, N fertilization is applied to the crop, which may further suppress the plant-rhizobium symbiosis, often along with P fertilizer, which is known to suppress the mycorrhizal symbiosis. On the other hand, beans are considered promiscuous and known to nodulate with many different rhizobial species as *Rhizobium etli* Segovia et al. 1993 [former *Rhizobium leguminosarum* bv. *phaseoli* (Frank 1879) Frank 1889 AL], *Rhizobium tropici* Martínez-Romero et al. 1991, *R. gallicum* Amarger et al. 1997, *R. giardinii* Amarger et al. 1997, *R. lusitanum* Valverde et al. 2006, *R. phaseoli* Dangeard 1926AL, *R. azibense*

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Mnasri et al. 2014, *R. freirei* Dall'Agnol et al. 2013, *R. mesoamericanum* López-López et al. 2011, *Ensifer meliloti* (former *Sinorhizobium meliloti*) (Dangeard 1926) Young 2003, *S. americanum* corrig. Toledo et al. 2004, and *Bradyrhizobium* sp. (Cao et al. 2014; Dall'Agnol et al. 2013; López-López et al. 2012; Mnasri et al. 2012, 2014; Zurdo-Piñeiro et al. 2009). Selection of effective native rhizobia was found to improve bean N nutrition through N_2-fixation at least at the same level as N fertilization (Akter et al. 2014; Giller and Cadisch 1995; Hardarson et al. 1993; Rahmani et al. 2011; Rodriguez-Navarro et al. 2000).

The Prespa lakes plain, located at northwestern Greece, is a high-elevated, distal and isolated area participating in the EU Natura2000 network of protected areas (http://ec.europa.eu/environment/nature/natura2000/index_en.htm). It includes a National Park, declared in 1974, and is protected by the RAMSAR convention of wetlands. The area is famous for the production of climbing dry beans (*Phaseolus vulgaris* L. and *Phaseolus coccineus* L.) being one of the main bean producing areas in Greece. Bean crop is actually a monoculture with practically no crop rotation in the area. As a consequence, heavy N fertilization is applied to a vulnerable ecosystem with a high possibility of N leaching to the lakes. While the genetic diversity of the local bean landraces has been studied (Tertivanidis et al. 2008; Mavromatis et al. 2010), there are no studies evaluating the native rhizobia diversity which could be the first step for the enhancement of N_2-fixation in beans, as it has already been done for alfalfa (Embalomatis et al. 1994). Effective inocula could reduce the cost of bean cultivation and be environmentally beneficial (Graham et al. 2003), especially for such a sensitive area.

The aim of the present work was to provide an estimate of the genetic diversity of native bean rhizobia and the percentage of AMF root length colonization along with AMF spore abundance in fields in Prespa lakes plain. This is a prerequisite step to isolate and evaluate effective strains for inoculum development.

Materials and methods

Field sampling

During 2013 growing season, scattered fields were selected to cover most of the bean growing area of the plain. In total, 19 fields from 10 areas of the Prespa lakes plain sown to climbing dry beans (*P. vulgaris* L. and/or *P. coccineus* L.) were sampled. Six areas were sampled on 28 June 2013 and five on 29 July 2013. One area (Slatina-Laimos) was sampled at both times at the same fields (Table 2). At the first sampling, the plants in most fields of the area were at early growth stages, limiting the number of fields where plants were at bloom. Therefore, a month was allowed between samplings. The average size of local fields ranged from 0.3 to 3.0 ha. Of the 19 fields, 11 were sown to *P. vulgaris* (landrace Plaki Prespas), five were sown to white-seeded *P. coccineus* (landrace Giant) and three to colored *P. coccineus* (landrace colored Giant).

From each field, roots of bean plants were exposed with a spade, and rhizosphere soil, root and nodule samples were collected. Composite soil and root samples consisted of the roots of at least three plants.

Mycorrhizal presence, nodulation index and soil analysis

Root samples were stained for the measurement of root length colonization and spores of AM fungi were counted in 50 g soil using wet sieving (Sylvia 1994). For field nodulation evaluation, the nodulation index was used (Prévost and Antoun 2007).

The soil samples of the second sampling were air-dried and sieved through a 2-mm mesh, total organic carbon (OC) was determined by wet oxidation, pH in water (1:2.5), electrical conductivity (EC) was measured and soil NO_3-N was extracted with 1 M KCl and determined with ultraviolet spectrometry. Olsen-P was determined by the molybdenum blue-ascorbic acid method.

Bacterial isolation and plant nodulation assay

The nodule rhizobia were isolated using yeast extract mannitol agar with Congo red (Somasegaran and Hoben 1985) and repeated streaking on new plates. The isolates were checked with Gram stain (rejecting Gram+ isolates) and they were further screened with a plant inoculation assay recording formation of nodules as positive or negative initially with *P. vulgaris* and the positives were further verified with *P. coccineus*. Surface sterilized *P. vulgaris* or *P. coccineus* bean seeds (10% commercial bleach for 5 min followed by several washes with sterile distilled water) were pre-germinated in 250 ml styrofoam/polystyrene cups filled with autoclaved sand:vermiculite (1:1) mixture and inoculated with 1 ml of rhizobial isolate culture grown in yeast-mannitol broth with ~ 10^9 cells. Non inoculated controls were also included. Plants were grown in a growth room with a 12 h photoperiod, day/night temperatures of 30 °C/20 °C, under Sylvania Grolux F36W/Gro fluorescent lamps. The plants were watered with N-free plant nutrient solution as needed. Plants were checked for nodulation 30 d after inoculation. Isolates were maintained in slants at 4 °C.

Restriction fragment length polymorphism analysis and bacteria identification

A procedure similar to Rahmani et al. (2011) was used for further screening of 72 successfully nodulating isolates, using PCR amplification of the 16S-ITS-23S followed by restriction fragment length polymorphism analysis (RFLP). Bacterial DNA was isolated from rhizobial isolates using the kit Nucleospin Tissue (Macherey-Nagel Düren, Germany) and according to the manufacturer's instructions. PCR was performed using the primers FGPS1490 and FGPL132 (Laguerre et al. 1996) in 50-µl reactions each containing 5 µl of 5 × PCR buffer, 1.5 mM $MgCl_2$, 10 pmol of each primer, 200 µM of each dNTP and 2 U of polymerase (KAPATaq, Kapabiosystems, Boston, USA). Thermocycling conditions included an initial denaturation at 95 °C for 3 min, 34 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 2 min) with a final extension of 72 °C for 2 min. Restriction was carried out using the *Hae*III and *Msp*I (New England Biolabs, Ipswich, Massachusetts, USA) endonucleases in 50-µl reactions where 10 µl of the PCR product were digested with 1 µl (10 U) enzyme at 37 °C for 2 h. The fragments were visualized on agarose gel and bands from undigested PCR products from isolates with unique pattern were excised from agarose gels, cleaned with Ultra Clean GelSpin DNA Extraction Kit (MoBio, Carlsband, CA, USA) following the manufacturer's instructions and sequenced using both primers mentioned above or only FGPL132.

PyElph (Pavel and Vasile 2012) was used for band clustering and generation of a binary matrix that was used for an UPGMA cluster analysis dendrogram construction using PAST v. 3.20 software with the Dice similarity index. A maximum likelihood phylogenetic tree of the portion of the ITS amplicon spanning from the tRNA-Ile to the start of the 23S rRNA was constructed using the PhyML v. 3.1 (Guindon and Gascuel 2003) algorithm under the optimal evolutionary model calculated by JModelTest v. 2.1.10 (Darriba et al. 2012). The Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT) was used for branch support. Alignments were constructed using MAFFT v. 7 (Katoh and Standley 2013). Sequences were deposited in GenBank genetic sequence database under the accession numbers MK590264, MK590266–MK590289.

Results

Soil analysis

The soil analysis of the fields sampled at the second sampling date presents the variability of the soils in the area in pH (5.5–7.9) and organic carbon (0.08–1.51%) and the heavy fertilization with overall high N and P levels (Table 1).

Mycorrhizal presence and field nodulation index

The fields sampled were sown at different dates with different species and varieties. Mycorrhizal fungal root length colonization ranged from nil to high (0–86%), depending on the field and sampling date, while AMF spore numbers were generally low (0–25 spores 100 g^{-1} soil) with a low number of morphotypes present per field (1–4) and for the area (< 10) (Table 2). The field with zero colonization had the youngest plants sampled, at about 15 cm height. All the other fields had plants at flowering.

High variability was present in nodulation between fields (Table 2). Only one field, on the second sampling date (29 July 2013), had very high nodulation with red nodules while others barely had any nodules present (Table 2).

Table 1 Some soil characteristics of the sampled fields in Prespa lakes plain

Site	Soil type	Clay	Silt (%)	Sand	OC (%)	pH	EC (mS cm^{-1})	Olsen-P (mg kg^{-1})	NO_3-N (mg kg^{-1})
PYL	L	24.2	41.8	34.0	0.37	7.54	0.79	130.2	280.1
PYL	L	19.2	36.8	44.0	0.61	7.90	0.22	130.0	27.3
PYL	L	25.2	31.8	43.0	0.89	7.94	0.41	117.3	110.0
PYL	SL	19.2	25.8	55.0	1.51	7.43	0.72	133.6	213.6
SLL	SL	14.2	15.8	70.0	0.99	7.02	0.78	65.8	263.5
SLL	L	17.2	30.8	52.0	0.35	7.03	0.49	52.1	120.9
KAR	SL	15.2	16.8	68.0	0.08	6.08	0.38	45.2	61.4
KAR	SL	17.2	18.8	64.0	0.40	5.91	0.48	32.7	115.8
KAR	SCL	23.2	24.8	52.0	0.75	5.57	0.41	14.9	129.6
MIK	SL	17.2	22.8	60.0	1.25	7.47	0.24	131.8	38.2
MIK	L	26.2	41.8	32.0	0.68	6.88	0.45	50.1	63.3

L loam, *SL* sandy loam, *SCL* sandy clay loam, *OC* organic carbon. For area abbreviations, see Table 2

Table 2 Areas, coordinates, presence of AMF and nodulation index of bean roots sampled from fields in Prespa lakes plain

Date	Area	Code	Coordinates	Bean type	Spores (50 g soil)	Morphotypes (number)	Colonization (%)	NI
28/6/13	Laimos 5th axis	LAI	40°49′8N, 21°06′6E, 855 m	*P. vulgaris*	0	0	54.8	4
		LAI	40°49′8N, 21°06′6E, 855 m	*P. vulgaris*	0	0	0.0	6
	Ergotaxio	ERG	40°49′7N, 21°07′3E, 864 m	*P. coccineus* (W)	6	3	72.5	2
	Slatina 9th axis	SLA	40°49′2N, 21°06′5E, 857 m	*P. vulgaris*	5	3	78.0	4
	Slatina-Laimos 1st axis	SLL	40°49′6N, 21°07′3E, 865 m	*P. coccineus* (C)	0	0	0.0	8
		SLL	40°49′6N, 21°07′3E, 865 m	*P. vulgaris*	2	1	47.2	8
	Opalia	OPA	40°48′6N, 21°07′1E, 864 m	*P. vulgaris*	1	1	32.2	8
	Orman	ORM	40°48′0N, 21°07′1E, 865 m	*P. coccineus* (W)	5	2	45.5	4
		ORM	40°48′0N, 21°07′1E, 865 m	*P. vulgaris*	1	1	17.2	4
	Graista	GRA	40°47′0N, 21°07′4E, 863 m	*P. vulgaris*	5	3	11.1	8
29/7/13	Pyli	PYL	40°46′2N, 21°02′7E, 859 m	*P. coccineus* (W)	10	4	86.4	8
		PYL	40°46′2N, 21°02′7E, 859 m	*P. vulgaris*	3	2	9.0	2
		PYL	40°46′2N, 21°02′7E, 859 m	*P. vulgaris*	25	3	22.5	12
		PYL	40°46′6N, 21°02′7E, 860 m	*P. coccineus* (C)	3	2	48.0	4
	Slatina-Laimos 1st axis	SLL	40°49′6N, 21°07′3E, 865 m	*P. vulgaris*	1	1	58.4	18
		SLL	40°49′6N, 21°07′3E, 865 m	*P. coccineus* (C)	4	2	56.4	4
	Karyes	KAR	40°45′4N, 21°08′9E, 909 m	*P. coccineus* (W)	1	1	49.0	4
		KAR	40°45′4N, 21°08′9E, 913 m	*P. vulgaris*	1	1	52.0	12
		KAR	40°45′0N, 21°08′3E, 880 m	*P. coccineus* (C)	1	1	38.0	6
	Mikrolimni	MIK	40°45′1N, 21°07′8E, 863 m	*P. vulgaris*	5	2	18.7	12
		MIK	40°45′1N, 21°07′6E, 861 m	*P. coccineus* (W)	3	1	11.4	12

Fields with the same coordinates were either next to each other or separated by a rural road

NI nodulation index, *W and C* white- and colored-seeded *P. coccineus*, respectively

Plant nodulation assay and genetic diversity of bean rhizobia

The nodulation assay showed that all isolates that nodulated *P. vulgaris* also nodulated *P coccineus* (Table 3). There were no nodules in the non-inoculated controls. Due to the procedure followed, it is possible that at screening, isolates nodulating *P. coccineus* but not *P. vulgaris* were discarded.

Variability appeared in the length of the PCR product that spanned from 950 to 1300 bp, giving a unique band at either 1100 or 1200 bp for most isolates, while several isolates either gave smaller size bands (but larger than 500 bp) or had multiple bands (Supplementary material S1 and S2). The length of the sequenced DNA was 538–1165 bp from 26 isolates, of which two had the same restriction band pattern, and two had two bands each sequenced (Table 4).

Cluster analysis gave up to 34 ITS-RFLP patterns from 47 isolates, to which another 11 isolates, that had unique position and number of bands at PCR, may be added for a total of 44 patterns from 72 isolates. Restriction with enzyme *Hae*III resulted in 12 clusters (Fig. 1a), while with the enzyme *Msp*I there were 22 (Fig. 1b). However, the phylogenetic tree (Fig. 2) showed that isolates from different clusters had high phylogenetic similarity. For example, LAI3 and LAI4 were phylogenetically very close (Fig. 2), but were found in different clusters with both enzymes; LAI3 was in clusters VIII (Fig. 1a) and XXII (Fig. 1b) and LAI4 in clusters III (Fig. 1a) and I (Fig. 1b). Some isolates in the phylogenetic tree (Fig. 2) were phylogenetically close to uncharacterized rhizobia (clade of SLA1, GRA1, LAI1C, MIK4B, clade of LAI5, LAI4, LAI3, SLL12 and clade of MIK1). The closest matches included great geographic distribution and a variety of host plants (Table 4).

Discussion

With ca. 1000 ha seeded to beans, the Prespa lakes plain is a relatively small area, with variable topography and slopes ranging from 0 to 10% to more than 35% in the surrounding hilly areas (Kosmas et al. 1990). The Entisols that predominate the agricultural land have 69 soil series in the area. In addition, the average field size of 0.3–3.0 ha implies variation in management, although beans were practically grown without any crop rotation. The common practice in the region is to heavily fertilize with 300–1000 kg ha^{-1} of ammonium phosphate before sowing and 300–500 kg ha^{-1} of mixed fertilizer (11–15–15) as top dressing (Kosmas

Table 3 Site isolates that were successful in nodulation, their original host in the sampled fields in Prespa lakes plain, and results of the plant nodulation assay

Site	Isolates	Isolated from	Nodulation of	
			P. vulgaris	*P. coccineus* (W)
Laimos 5th axis	LAI1–9	*P. vulgaris*	+	+
Ergotaxio	ERG1–3	*P. coccineus* (W)	+	+
Slatina 9th axis	SLA1–6	*P. vulgaris*	+	+
Slatina-Laimos 1st axis	SLL1–3, 6, 7, 11, 14	*P. coccineus* (C)	+	+
	SLL4, 5, 8, 9, 12	*P. vulgaris*	+	+
Opalia	OPA1, 2	*P. vulgaris*	+	+
Orman	ORM1–6	*P. coccineus* (W)	+	+
Graista	GRA1–5	*P. vulgaris*	+	+
Pyli	PYL2, 4, 10, 11	*P. vulgaris*	+	+
	PYL8, 9, 12	*P. coccineus* (W)	+	+
	PYL3, 5, 6, 7	*P. coccineus* (C)	+	+
Karyes	KAR1–3, 11	*P. coccineus* (W)	+	+
	KAR4, 5, 10	*P. vulgaris*	+	+
	KAR6–9	*P. coccineus* (C)	+	+
Mikrolimni	MIK1–3, 5	*P. vulgaris*	+	+
	MIK4, 6, 7	*P. coccineus* (W)	+	+

W and C white- and colored-seeded *P. coccineus*, respectively

et al. 1990). Heavy fertilization is a plausible explanation for the overall low nodulation index, although in most of the fields the nodules were present. In Tunisia, with more than 100 kg N ha^{-1} applied, nodules were rarely found and were ineffective (Aouani et al. 1997), while almost no nodulation was reported from fields in Egypt (Elbanna et al. 2009).

The heavy fertilization practiced in Prespa lakes plain was expected to lead to low mycorrhizal presence. High soil P levels are generally known to inhibit the AM symbiosis and this may also be the case for high N levels (Hayman 1986). However, in most fields, the AMF colonization was still considerable indicating that the arbuscular mycorrhizal symbiosis may also be important for well-fertilized crops (Gryndler et al. 1989; Hayman et al. 1976; Miller et al. 1995). On the other hand, the AMF spore numbers and morphotypes were very low. In a limited number of fields, the very low AMF spore number, along with 10–20% root length colonization, suggested a low number of AMF propagules and in such cases application of AMF inoculum might prove beneficial.

Despite the isolation and restriction of the Prespa lakes plain, which contribute to genetic isolation (Van Cauwenberghe et al. 2014), considerable variation in bean rhizobia was found. Many isolates gave more than one band at PCR, however, this has been observed before and is explained by the existence of many copies of rRNA operons and polymorphism (Haukka et al. 1996; Rahmani et al. 2011). In addition, some clusters had all the isolates originating from the nodules of one bean species (e.g., Fig. 1a, clusters II, III, VI, VII, IX, XI, XII). However, there were only few isolates in each cluster and therefore there was no indication of diversification of isolates with bean species. On the other hand, all the isolates did nodulate both species in plant inoculation assays. More diversification of rhizobia may be expected with time in areas where bean is cultivated, and this may be enhanced with diversification in soil (Cao et al. 2014), climatic parameters (Adhikari et al. 2013) and presumably this is accelerated by monoculture. Bean rhizobia diversity has been studied in much larger areas, such as Iran (Abbaszadeh-Dahaji et al. 2012; Rahmani et al. 2011), Nepal (Adhikari et al. 2013), China (Cao et al. 2014; Wang et al. 2016), Jordan (Tamimi 2002), Egypt (Elbanna et al. 2009), Ethiopia (Aserse et al. 2012), Tunisia (Mnasri et al. 2007; Mhamdi et al. 1999), northern Spain (García-Fraile et al. 2010), Portugal (Valverde et al. 2006), Austria (Sessitch et al. 1997), Argentina (Anguilar et al. 2006), Brazil (Oliveira et al. 2011; Andrade et al. 2002), Ecuador, Mexico (Bernal and Graham 2001), and Chile (Baginsky et al. 2015; Junier et al. 2014). The biogeodiversity of rhizobia in the above-mentioned areas indicated no overall relationship between the geographical origin of rhizobia isolates and soil/climatic parameters.

Some sequence observations are more consistently related to particular soil/climatic conditions. In the Shaanxi Province of China, the diversity of rhizobia was related to moisture, temperature, intercropping, plow layer thickness and soil potassium levels (Wang et al. 2016). Others found that *R. tropici* prevails in acidic soils (Andrade et al. 2002; Baginsky et al. 2015), and this was the case in the present

Table 4 Rhizobia strains isolated from root nodules of *Phaseolus vulgaris* and *P. coccineus* in Prespa lakes plain, and their closest matches from GenBank (according to BLAST results) max score

Isolate (accession number)	Closest match (accession number)	Closest match host/site	References	Identity (%)	Length (bp)
LAI1-bandC (MK590271)	*Rhizobium leguminosarum* strain LPB0205 (GQ863516)	*Phaseolus vulgaris*/Northern Spain	García-Fraile et al. (2010)	99	1048
LAI2-bandA (MK590272)	*Agrobacterium tumefaciens* strain Ach5 (CP011246)	*Achillea ptarmica*/California	Huang et al. (2015)	99	1152
LAI2-bandB (MK590273)	*A. tumefaciens* strain Ach5 (CP011246)	*A. ptarmica*/California	Huang et al. (2015)	98	1150
LAI3 (MK590274)	*Rhizobium* sp. Mug-12 (AB740443)	*P. vulgaris*/subhumid temperate regions in Nepal	Adhikari et al. (2013)	99	716
LAI4 (MK590275)	*Rhizobium* sp. Mug-12 (AB740443)	*P. vulgaris*/subhumid temperate regions in Nepal	Adhikari et al. (2013)	99	1034
LAI5 (MK590276)	*Rhizobium* sp. Mug-12 (AB740443)	*P. vulgaris*/subhumid temperate regions in Nepal	Adhikari et al. (2013)	99	819
ERG1 (MK590266)	*Sinorhizobium meliloti* strain KH35c (CP021825)	*Medicago truncatula*/France	Nelson et al. (2018)	99	1256
ERG2 (MK590267)	*Rhizobium* sp. Kim 5(CP021124)	*P. vulgaris*/Idaho, USA	Santamaría et al. (2017)	100	416
SLA1 (MK590283)	*Rhizobium* sp. RPVR24 (GQ863510)	*P. vulgaris*/Northern Spain	García-Fraile et al. (2010)	99	855
SLA2 (MK590284)	*Rhizobium tropici* strain CFN299 (DQ785473)	*P. vulgaris*/Portugal	Valverde et al. (2006)	100	720
SLL2 (MK590285)	*R. mongolense* strain CCBAU01546 isolate 232 (EU418362)	*Astragalus* spp./highly fertilized soils in China	Yan et al. (2016), Wei et al. (2008)	99	1143
SLL3 (MK590286)	*R. mongolense* strain CCBAU01546 isolate 232 (EU418362)	*Astragalus* spp./highly fertilized soils in China	Yan et al. (2016), Wei et al. (2008)	99	749
SLL4 (MK590287)	*Azospirillum brasilense Sp245* (*CP022262*)	*Triticum* sp./Brazil	(Unpublished)	87	657
SLL5 (MK590288)	*Rhizobium* sp. LCS0401 (GQ863513)	*P. vulgaris*/Northern Spain	García-Fraile et al. (2010)	96	576
SLL12 (MK590289)	*Rhizobium* sp. Mug-12 (AB740443)	*P. vulgaris*/subhumid temperate regions in Nepal	Adhikari et al. (2013)	100	499
ORM1 (MK590280)	*R. leguminosarum* strain RVS11 (FJ596012)	*Vicia sativa*/Riego de la Vega (León), Spain	Álvarez-Martínez et al. (2009)	92	1167
ORM2 (MK590281)	*Rhizobium* sp. RPVR24 (GQ863510)	*P. vulgaris*/Northern Spain	García-Fraile et al. (2010)	99	813
GRA1 (MK590268)	*Rhizobium* sp. RPVR24 (GQ863510)	*Phaseolus vulgaris*/Northern Spain	García-Fraile et al. (2010)	99	813
PYL3 (MK590282)	*R. gallicum* bv. *gallicum* R602 (CP006877)	*P. vulgaris*/agricultural field with no beans grown for 5 years, Maine et Loire, France	Bustos et al. (2017)	99	586
KAR1 (MK590269)	*R. tropici* strain CFN299 (DQ785473)	*P. vulgaris*/Portugal	Valverde et al. (2006)	100	882
KAR2 (MK590270)	*Rhizobium* sp. RPVR04 (GQ863509)	*P. vulgaris*/Northern Spain	García-Fraile et al. (2010)	92	892
MIK1 (MK590277)	*Rhizobium* sp. Trr-4 (AF510892)	*Trifolium repens*/Korea	Kwon et al. (2005)	99	876
MIK2 (MK590264)	*Rhizobium sp.* I312(AB529841)	*Lathyrus japonicus*/Japan	Aoki et al. (2010)	100	642
MIK3 (MK590278)	*Rhizobium* sp. LCS0401 (GQ863513)	*P. vulgaris*/Northern Spain	García-Fraile et al. (2010)	99	867

Table 4 (continued)

Isolate (accession number)	Closest match (accession number)	Closest match host/site	References	Identity (%)	Length (bp)
MIK4-B (MK590279)	*R. leguminosarum* strain LPB0205 (GQ863516)	*P. vulgaris*/Northern Spain	García-Fraile et al. (2010)	98	630

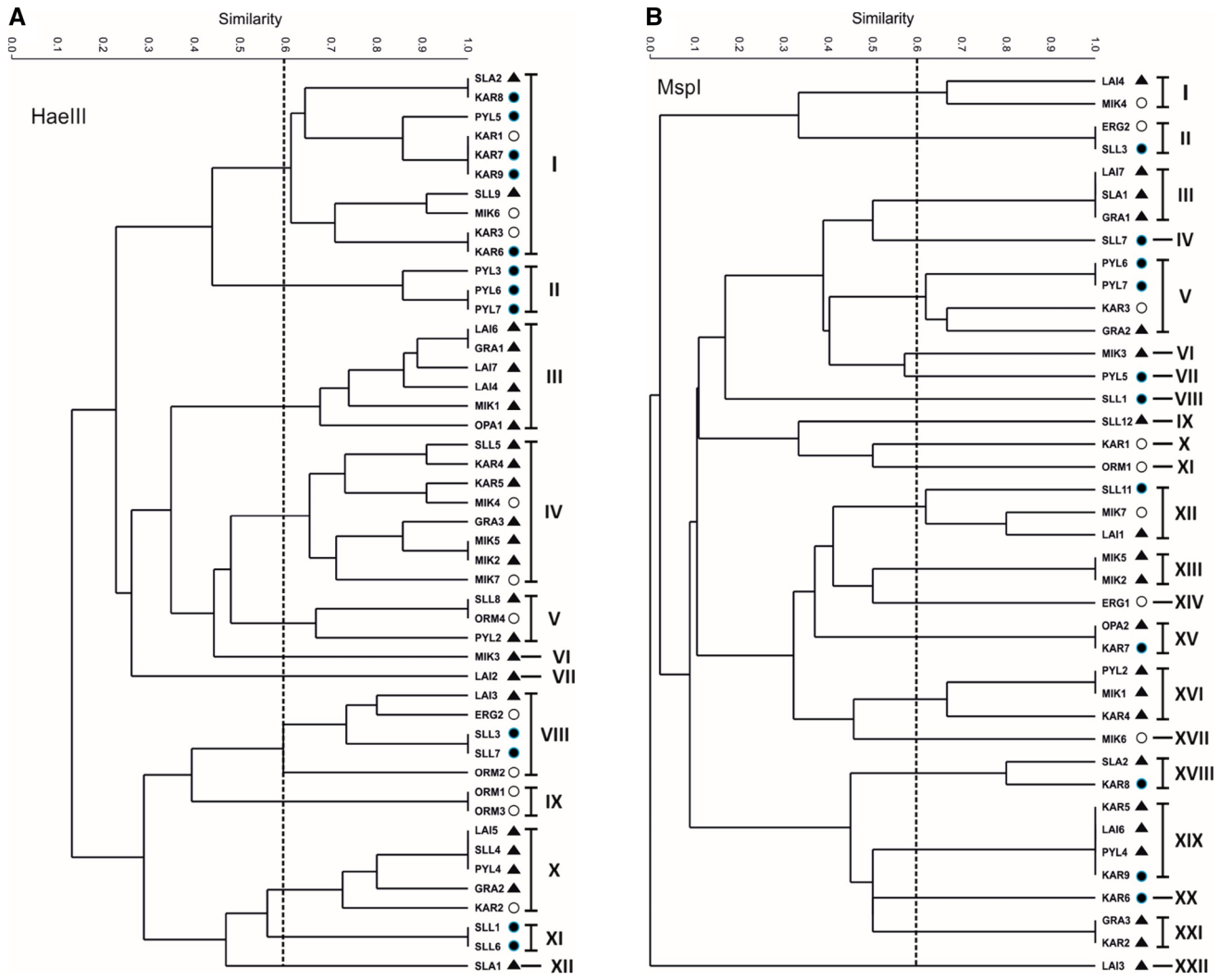

Fig. 1 Dendrogram based on the UPGMA cluster analysis of Dice index of normalized RFLP patterns of 16S–23S ITS with enzymes A: *Hae*III, B: *Msp*I, showing the genetic relationships among the rhizobial isolates. The branch length is proportional to the number of substitutions per site. Isolates originated from open circles = white *P. coccineus*, closed circles = colored *P. coccineus*, triangles = *P. vulgaris*

study where Kar1, phylogenetically close to *R. tropici*, was isolated from an area with acidic pH. In Nepal, *R. etli* was limited to semiarid temperate climate with alkaline soils and *R. leguminosarum* in temperate climate with slightly acidic to neutral soils (Adhikari et al. 2013). High soil organic matter was related to *R. etli* in south-central Chile, where *R. leguminosarum* was present in all soil types (Baginsky et al. 2015); *R. etli* was also found in salt stressed areas (Mnasri et al. 2007). *Rhizobium mongolense*-related strains from *Astragalus* spp. L. in China were related to heavily fertilized soils, noting that some rhizobia are more capable to nodulate under fertilization (Caballero-Mellado and Martínez-Romero 1999). In our study, where all the fields were heavily fertilized and actually without climate variation, most of the isolates were related to *R. leguminosarum*, but some were related with *R. etli* and *R. mongolense.*

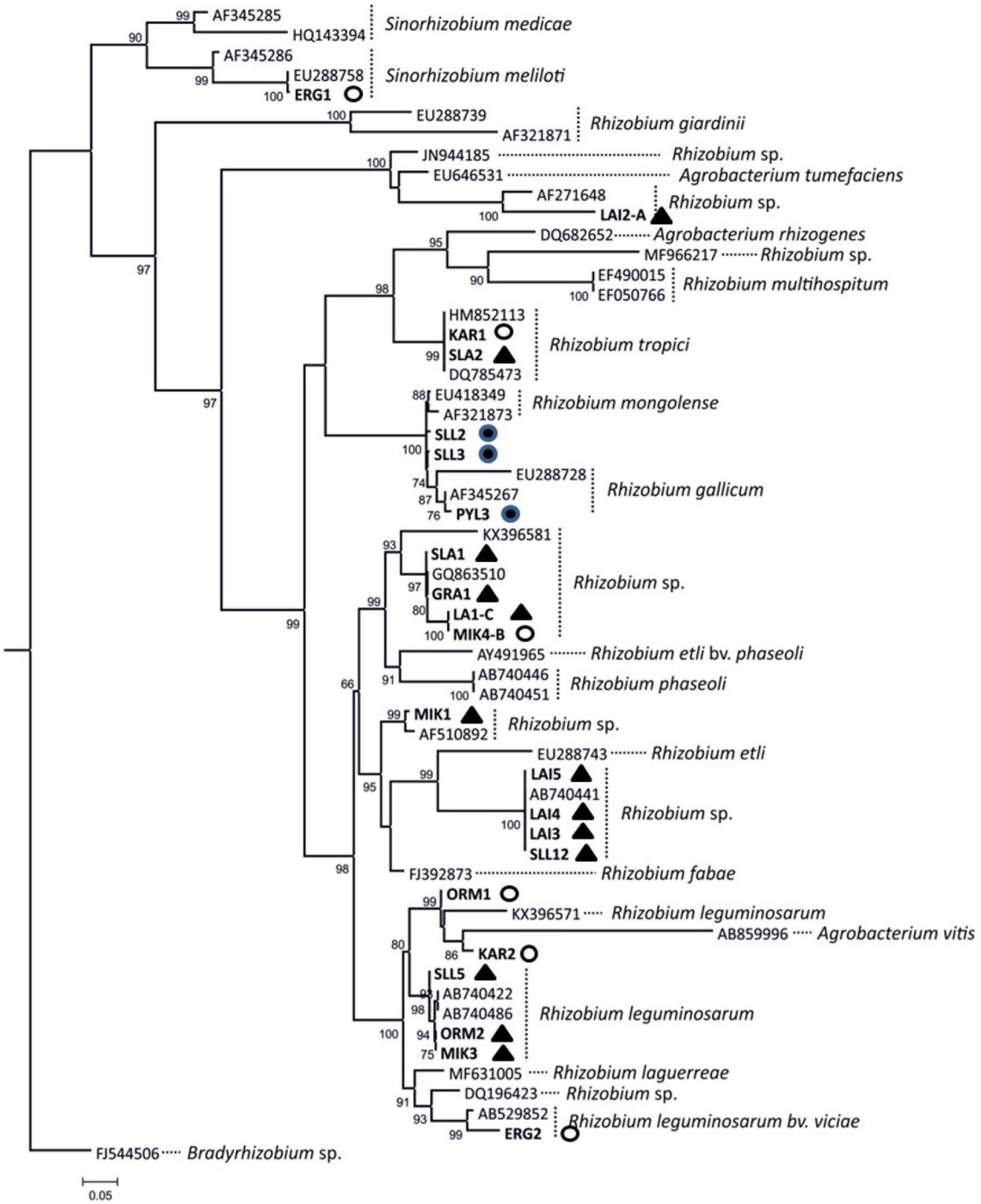

Fig. 2 Phylogenetic analysis of the 16S-23S internal transcribed spacer (ITS) amplicon sequence, spanning from the tRNA-Ile to the start of the 23S rRNA from the Prespa isolates and rhizobial references showing the relationships between the strains evaluated in this study and other type or reference strains from closely related species. GenBank accession numbers are indicated. *Numbers above nodes* represent the Shimodaira–Hasegawa approximate likelihood ratio scores (SH-aLRT) support for clades. The bar below the tree indicates substitutions per site. Sequences from this study are indicated in bold. Isolates originated from open circles = white *P. coccineus*, closed circles = colored *P. coccineus*, triangles = *P. vulgaris*

It has been hypothesized that *R. etli*, prevalent in the areas of bean origin, was spread with bean seeds, however, lateral gene transfer to the local *R. leguminosarum* takes place in areas where bean is cultivated (Herrera-Cervera et al. 1999; Rodriguez-Navarro et al. 2000; Pérez-Ramírez et al. 1998). In Spain, it was shown that *R. etli* was found in the southern

area, where bean is not grown, and *R. leguminosarum* in the north, where bean is cropped (García-Fraile et al. 2010). Reversely, it has been hypothesized that *R. leguminosarum*, with a presumed origin in Europe, was spread to the rest of the world with the seeds of *Vicia* species (Álvarez-Martínez et al. 2009). Similarly, in Prespa lakes plain, where beans are grown with no rotation, while *R. etli* was present, *R. leguminosarum*-related isolates were prevalent. Hou et al. (2009) noted that Tibetan legumes and rhizobia were not highly specific for symbiotic partners, which may be the case in the present study as well.

Strains related to *S. meliloti*, *R. gallicum* and *Agrobacterium tumefaciens* (Smith & Townsend) Conn were also found. They all have also been reported from bean fields in Brazil (Andrade et al. 2002), while *S. meliloti* was found in Tunisia where it was particularly tolerant to NaCl and was reported as a novel biovar, bv. *mediterranense* (Mnasri et al. 2007). In addition, *Agrobacterium* spp. isolates in Shaanxi, China did carry the *nodC* and *nifH* genes and could form nodules with bean (Wang et al. 2016). Regarding *R. gallicum*, it was found to be the prevalent bean rhizobium in France (Amarger et al. 1997) and was also found on salty soils in Tunisia (Mhamdi et al. 1999; Mnasri et al. 2007) and Korea (Kwon et al. 2005). Moreover, there was a significant number of isolates falling outside the already characterized species of genus *Rhizobium*.

Among local rhizobial populations, there may be great variation in efficiency that may also vary with bean variety (Akter et al. 2014; Graham et al. 2003; Hardarson et al. 1993). Prevalence of competitive ineffective strains may prevent the success of inoculation with effective strains (Thies et al. 1992). Selection of effective, competitive local strains is a key factor to increase nodulation and N_2-fixation in beans (Graham et al. 2003). In the present study, it was shown that there was high diversity among the local rhizobial population, which could likely allow such a selection. For the ecologically sensitive Prespa lakes plain this is not only of financial importance, but it has also high environmental significance in terms of limiting N discharge to the lakes.

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