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Nodules of *Medicago* spp. Host a Diverse Community of Rhizobial Species in Natural Ecosystems

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Abstract: Biological nitrogen fixation by rhizobia-nodulated legumes reduces the dependence on synthetic nitrogen fertilizers. Identification of locally adapted rhizobia may uncover economically valuable strains for sustainable agriculture. This study investigated the diversity and symbiotic potential of rhizobia associated with three *Medicago* species from Eastern Romania's ecosystems. Phenotypic screening ensured that only rhizobial species were retained for molecular characterization. 16S rDNA sequencing clustered the isolates into four distinct groups: *Sinorhizobium meliloti*, *Sinorhizobium medicae*, *Rhizobium leguminosarum*, and *Mesorhizobium* spp. The chromosomal genes (*atpD*, *glnII*, *recA*) and *nifH* phylogenies were congruent, while the *nodA* phylogeny grouped the *Mesorhizobium* spp. isolates with *R. leguminosarum*. *Medicago sativa* was the most sampled plant species, and only *S. meliloti* and *R. leguminosarum* were found in its nodules, while *Medicago falcata* nodules hosted *S. meliloti* and *Mesorhizobium* spp. *Medicago lupulina* was the only species that hosted all four identified rhizobial groups, including *S. medicae*. This study provides the first report on the *Mesorhizobium* spp. associated with *M. falcata* nodules. Additionally, *R. leguminosarum* and two *Mesorhizobium* genospecies were identified as novel symbionts for *Medicago* spp. Comparative analysis of *Medicago*-associated rhizobia from other studies revealed that differences in 16S rDNA sequence type composition were influenced by *Medicago* species identity rather than geographic region.

Keywords: *Medicago* spp.; root nodules; rhizobia diversity; molecular taxonomy

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Citation: Stefan, A.; Van Cauwenberghe, J.; Rosu, C.M.; Stedel, C.; Chan, C.; Simms, E.L.; Iticescu, C.; Tsikou, D.; Flemetakis, E.; Efrose, R.C. Nodules of *Medicago* spp. Host a Diverse Community of Rhizobial Species in Natural Ecosystems. *Agronomy* **2024**, *14*, 2156. https://doi.org/10.3390/agronomy14092156

Academic Editors: Franco Magurno and Zoltán Kende

Received: 22 August 2024
Revised: 18 September 2024
Accepted: 19 September 2024
Published: 21 September 2024

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1. Introduction

Rhizobia are motile, naturally occurring rod-shaped soil bacteria. Following a complex exchange of signaling molecules between the bacteria and plants of the legume family and activation of subsequent cellular pathways, the bacteria can enter plant root cells and initiate the formation of specialized organs on the roots called nodules [1–3]. In these nodules, the bacteria can differentiate and are capable of fixing atmospheric nitrogen into ammonia, which they transfer to the host plant in exchange for carbon-containing molecules and shelter [4,5]. Biological nitrogen fixed by this bacteria-plant symbiosis [6] contributes large amounts of nitrogen to agricultural systems [7]. Soils with poor nitrogen-fixing rhizobial communities can be supplemented by inoculation with superior nitrogen-fixing rhizobia. The application of rhizobial inoculants to crop fields can reduce the amount of synthetic nitrogen fertilizers, can reduce emissions of the potent greenhouse gas N_2O by

converting it to nitrogen [8], can improve crop productivity [9], nutrient mobilization and solubilization [10].

Crop rotation every few years with legume species and intercropping practices locks the nitrogen molecules into plant structures, thus reducing nitrogen depletion from the soil [11–13]. Some legume species (like alfalfa and clovers) are specifically planted to be incorporated into the soil while still green ('green manuring') to benefit subsequent crops [14–16]. The isolation and characterization of indigenous rhizobia can pave the way for their use as inoculants [17] and can lead to the discovery of strains of potential economic value. Native rhizobial strains, tolerant to local soil conditions, can be more efficient in nitrogen fixation and outcompete commercial inoculants [18–22].

One way of characterizing the rhizobial community is by sequencing multiple chromosomal and plasmid loci, a method that has been previously used in *Medicago* rhizobia studies [23–25]. This multilocus approach has been used extensively in rhizobial diversity studies in various legume species [26,27] across different regions and soil conditions, from subpolar soils [28] to arid desert soils [29] and in the characterization of indigenous rhizobia [30–32].

Among the most economically important legumes in temperate regions are those of the genus *Medicago*, colloquially known as medics. Medics comprise more than 80 species, both perennial and annual, with the main diversity region being the Mediterranean basin [33]. Used primarily for livestock fodder, medics can be cultivated or can grow wildly in a variety of environments, such as pastures, rock quarries, and urbanized environments, and are resilient to a broad range of stress factors [34–36]. The rhizobia most commonly found in medic nodules are *Sinorhizobium meliloti* and *Sinorhizobium medicae* [24,25,37,38], but some *Medicago* species can host a more diverse rhizobial community [39,40].

There are ten species of naturally occurring *Medicago* plants in Romania [41], with *M. sativa* being the most widely cultivated legume in terms of cultivated area [42]. In this study, rhizobia were isolated from nodules of three species of *Medicago* sampled from diverse landscapes in Eastern Romania: the perennial *Medicago sativa* (alfalfa, lucerne) and *M. falcata* (yellow lucerne), and the annual *Medicago lupulina* (black medic). The goals were to identify and characterize indigenous, locally adapted rhizobia associated with these *Medicago* species capable of establishing efficient nitrogen-fixing symbiosis and to gain insights into how Medicago spp. and the collection areas could shape the rhizobial community.

2. Materials and Methods

2.1. Nodule Collection and Isolation of Bacteria

During the spring of 2013, naturally occurring and cultivated *Medicago* plants were sampled in the Eastern part of Romania. To our knowledge, the cultivated fields have no history of inoculation. Sample sites spanned a variety of environments and elevations in the Eastern Carpathian Mountains and the Moldavian Plateau (Figure 1). The plant populations were grouped into four regions, named from North to South: Rarău, Lespezi, Iași, and Oituz. Each plant population was marked with a capital letter, from A to M, for readability (Supplementary Table S1).

Intact nodules were collected from three *Medicago* species (*M. sativa*, *M. falcata*, *M. lupulina*), surface sterilized, and individually stored in 20% glycerol following a brief visual inspection of the nodules until subsequent processing. Only active nodules were collected, as determined by the pinkish color indicating the presence of leghaemoglobin. A total of sixty-one rhizobial strains were isolated from plant root nodules using standard protocols [43,44] and purified by repeated plating of single colonies on yeast-extract-mannitol agar (YMA) supplemented with 25 μg/mL Congo red [44]. Bacterial isolates were incubated at 28 °C for 3 to 5 days and evaluated for Gram reaction, colony morphology, and acid/alkaline reaction on YMA plates containing 25 μg/mL bromothymol blue [45]. Pure cultures were maintained on YMA slants at 4 °C or preserved in yeast mannitol broth (YMB) containing 20% (*v*/*v*) glycerol at −80 °C. The pure cultures are part of the Romanian

Microbial Culture Collection (RMCC) established at the Department of Experimental and Applied Biology, NIRDBS-Institute of Biological Research, Iași, Romania.

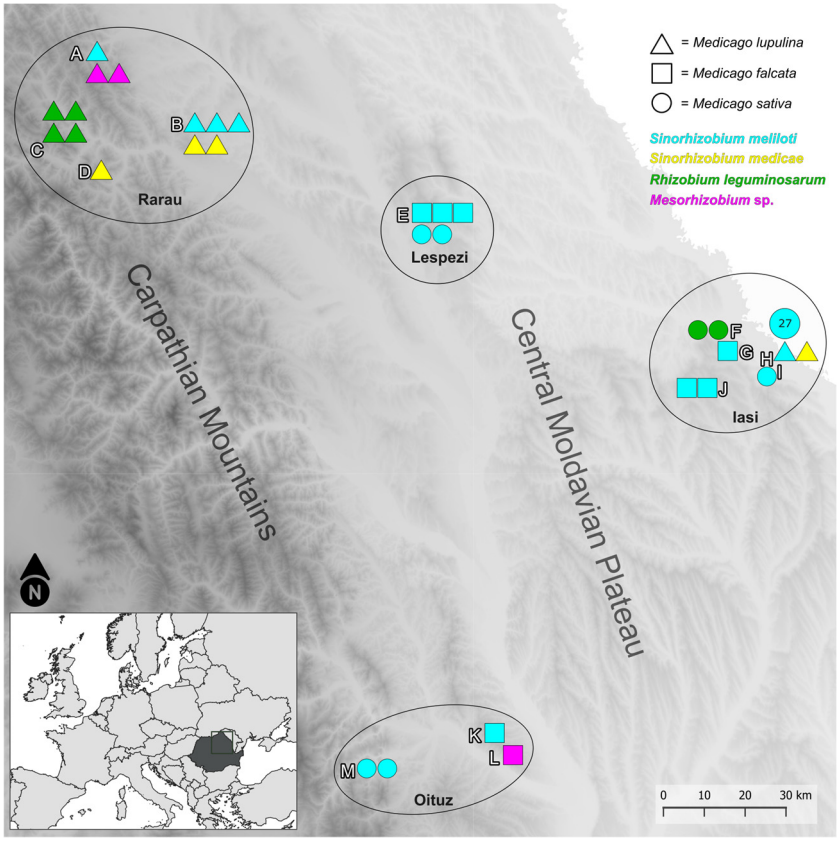


Figure 1. Map of the sampled plant populations. The shapes represent the three medic species (triangle = *Medicago lupulina*, square = *M. falcata*, circle = *M. sativa*), and the colors represent the four identified rhizobial groups (blue = *Sinorhizobium meliloti*, yellow = *S. medicae*, green = *Rhizobium leguminosarum*, magenta = *Mesorhizobium* spp.). Each symbol represents one rhizobial isolate, with the exception of plant population H, where the enlarged circle stands for 27 isolates. The square part on the inset map represents the sampling area. The ellipses represent the four geographic regions. The position of the plant population letter (A–M) represents the sampling point.

2.2. Phenotypic Characterization of Rhizobia

The rhizobial isolates and selected reference strains (*Sinorhizobium meliloti* LMG 6133, *S. meliloti* 1021, *Rhizobium leguminosarum* bv. *trifolii* LMG 8820 and *Mesorhizobium loti* MAFF 303099) were examined for several phenotypic features, according to Eaglesham's technique [46] and further analyzed by numerical taxonomy [47]. Salt tolerance and pH growth range were evaluated on agar plates containing 0.1, 0.2, 0.3, 0.4, 0.75, 1, 2, and 3.5% (*w*/*v*) NaCl or YMA medium buffered with 1 M HCl or 1 M NaOH to adjust the pH to 4.8, 5.8, 6.8, 7.8 and 8.8, respectively. Rhizobial growth sensitivity to varying temperatures was assessed on YMA plates incubated at 15, 28, 33, 37, and 42 °C. Intrinsic antibiotic resistance (IAR) assays were performed on YMA solid medium supplemented with the following antibiotics (µg/mL): chloramphenicol (30; 100), streptomycin (10; 25), gentamicin (5; 25), rifampicin (2; 20), tetracycline (1; 10), neomycin (5; 15), kanamycin (10; 20), ampicillin (20; 50), carbenicillin (50; 200), and nalidixic acid (50; 300). The choice of antibiotics and concentrations were adapted from Josey et al. [48] and Mpepereki et al. [49]. For each strain, bacterial growth was recorded from colony counts of plates inoculated with serial dilutions of each culture after incubating for 5 days at 28 °C. The physiological traits of the bacterial strains were coded 1 and 0 for positive and negative reactions, respectively, and the simple matching similarity coefficient (S_{SM}) was calculated to generate a similarity matrix. The

Unweighted Pair Group Method using Arithmetic Means (UPGMA) was performed for cluster analysis of phenotypic features [50] using MVSP–Multivariate Statistical Package v3.2 (Kovach Computing Services, Anglesey, Wales).

2.3. Nodulation Test

The nodulation ability of the rhizobial isolates on their original host was assessed as previously described [51]. Each rhizobial isolate was used to inoculate seeds from the *Medicago* species from which they were isolated. Briefly, batches of five surface-sterilized germinated seeds were transferred to individual pots containing sterile perlite and inoculated with 1 mL (1×10^8 cells/mL) of bacterial culture. Plants inoculated with strains *Sinorhizobium meliloti* 1021 and *S. meliloti* LMG6133 were used as a reference, and uninoculated plants were used as controls. The inoculated plants and controls were grown in strict conditions (18 h day/6 h night, 24 °C day/18 °C night cycles, 70% humidity) and watered every two days alternatively with N-free Hoagland solution and deionized water. Plants were harvested at 45 days post-inoculation, and the modulation and N-fixing abilities of the isolates were estimated by the number of pink nodules on the roots and the total dry weight of the plants.

2.4. DNA Extraction, Amplification and Sequencing

Pure cultures of rhizobial isolates were grown in liquid YMB and incubated at 28 °C for three days on a rotary shaker. Total genomic DNA was isolated from equal-sized aliquots of bacterial cultures. DNA isolation, PCR reactions, and sequencing were performed as described in detail elsewhere [51,52]. Six genetic markers were amplified, purified, and directly sequenced (CeMIA, Larissa, Greece) from each bacterial isolate: 16S rDNA, *atpD* (ATP synthase beta-chain), *glnII* (glutamine synthetase), *recA* (recombinase A), *nodA* (nodulation protein A), and *nifH* (nitrogen reductase).

2.5. Genetic Analyses

The raw chromatogram files were visually inspected and manually edited in Chromas v2.6.6 (Technelysium Pty Ltd., Brisbane, Australia). Alignments were assembled into contigs, and ambiguous regions were checked in CodonCode Aligner v3.7.1 (CodonCode Corporation, Centerville, MA, USA). Files were exported to fasta format, and basic sequence statistics were computed in DnaSP v6.12 [53]. To ensure that all isolates were indeed rhizobia, the 16S rDNA sequences were initially compared to GenBank [54] sequences using BLASTn [55]. If the top BLAST hits did not belong to putative rhizobial species, then the entire sequence set for that particular isolate was discarded. The most similar GenBank sequences were downloaded and included in the analyses to position isolates among known rhizobia. Identical sequences were collapsed into a single sequence type (ST), and the sequence types that contained a single sequence were denoted as singleton ST (sST). Phylogenetic and molecular evolutionary analyses were performed in MEGA X [56]. The phylogenies inferred separately from each gene sequence as well as from concatenated sequences of the *atpD*, *glnII*, and *recA* chromosomal genes were based on the Maximum Likelihood method using the best DNA substitution model. Bootstrap confidence levels were calculated for 1000 replicates.

The rhizobia obtained in the present study was compared with the previously described rhizobia associated with *Medicago* spp. across the world. Additional 16S rDNA sequences were obtained from studies found using the search term "*Medicago* rhizobi* diversity" on the Web of Science. A *Medicago* species × ST per sampling site matrix was constructed, as well as a *Medicago* species × rhizobia species per sampling site matrix. For each matrix, a Permutational Multivariate Analysis of Variance (PERMANOVA) based on Jaccard distances was performed using 999 permutations, with *Medicago* species and region as factors, in the *ecodist* [57] and *vegan* [58] packages in R v3.6.3 [59]. Differences among rhizobium communities were visualized by a Principal Coordinates Analysis (PCoA) with

ade4 [60] and *adegraphics* [61] R packages. The map was made using QGIS v3.28 [62]. Further visual edits to the map, graphs, and phylogenetic trees were made in Inkscape v1.2 [63].

2.6. Nucleotide Sequence Data

The GenBank accession numbers for the sequences reported in the present study are MT706727-MT706790 (for 16S rDNA); OM677466-OM677522 (for *atpD*); OM735848-OM735904 (for *glnII*); OM735905-OM735961 (for *recA*); OM970805-OM970861 (for *nodA*) and ON016017-ON016073 (for *nifH*). Accession numbers of the related reference strains are individually specified in the corresponding phylograms.

3. Results

3.1. Phenotypic Characterization and Nodulation of the Rhizobial Isolates

The phenotypic screening produced sixty-one rhizobial isolates that were Gram-negative, fast-growing, and acid-producing bacteria. After 3–5 days of incubation at 28 °C on YMA medium, most isolates formed white–opaque and mucoid-type colonies, slightly domed with smooth margins (*Sinorhizobium* sp.). Other colonies were small (<1 mm in diameter), translucent, with entire margins (*Mesorhizobium* spp.), and only a few isolates formed single colonies with diameters of 1–3 mm, white or creamy, gummy, smooth, raised, and circular with entire margins (*Rhizobium leguminosarum*).

Based on their physiological features (47 variables), the native rhizobial isolates and reference strains were distributed into two main clusters at a similarity level of 77% (Supplementary Figure S1 and Supplementary Table S2). The majority of the native isolates composed the largest cluster (I) and were separated into two subclusters (I.1 and I.2) at a similarity level of 87% due to differences in temperature tolerance. The majority of subcluster I.1 strains grew best at temperatures between 15 and 37 °C, while strains belonging to subcluster I.2 exhibited thermotolerance between 28 and 42 °C. All isolates could grow at pH values between 5.8 and 8.8 and tolerate 2.0% (*w*/*v*) NaCl, but only 10% of them grew at 3.5% (*w*/*v*) NaCl. Moreover, all isolates manifested multiple intrinsic antibiotic resistances (IAR) against low concentrations of antibiotic substances, except streptomycin (34%). Two isolates were placed separately due to low salt tolerance (maximum 0.75% NaCl; RMCC MS1312) or higher resistance to streptomycin (25 µg/mL; RMCC MS1011). Cluster II included twelve rhizobial isolates with the most diverse and divergent physiological features and were distributed into two subclusters at a similarity level of 81%. They all grew at temperatures between 15 and 33 °C and in a broad range of pH values (4.8 to 8.8). Cluster II.1 was the smallest, with only four isolates, including the reference strain *R. leguminosarum* bv. *trifolii* LMG 8820. These isolates and the reference strain tolerated a maximum concentration of 0.1% (*w*/*v*) NaCl, and the majority of the isolates (75%) showed resistance to low concentrations of all antibiotics tested. The rhizobial isolates that grouped together with *Mesorhizobium loti* MAFF 303099 reference strain (subcluster II.2.) tolerated NaCl concentrations up to 0.75% (*w*/*v*), with only *M. loti* MAFF 303099 reference strain growing at 2.0% (*w*/*v*) NaCl. Almost all isolates and strains (90%) exhibited high resistance to low concentrations of all antibiotics tested.

In the nodulation tests, all the isolated rhizobia induced the formation of pink nodules on the inoculated plant roots, and the plants were well developed (Supplementary Figure S2).

3.2. Molecular Characterization of Rhizobia

Following the phenotypic characterization of the bacteria, fifty-seven rhizobial isolates obtained from thirteen plant populations, either cultivated or wildly grown *Medicago sativa* (n = 34), *M. falcata* (n = 8), and *M. lupulina* (n = 15), were subsequently used for the genetic analyses.

The low-quality ends of the chromatograms were trimmed, and the sequences were corrected for frame-shift errors. The resulting working dataset consisted of six partially sequenced genetic markers that varied in length: 1345 bp (16S rDNA), 435 bp (*atpD*), 588 bp (*glnII*), 330 bp (*recA*), 168 bp (*nifH*), 580 bp (*nodA*). The closely affiliated GenBank

sequences were included in the analysis, and the whole sequence set was used to generate phylogenetic trees for each of the six loci.

The 16S rDNA sequences from our isolates clustered into three major groups: forty-eight isolates clustered in the *Sinorhizobium* group (I), six isolates clustered in the *Rhizobium leguminosarum* group (II), and three isolates clustered in the *Mesorhizobium* spp. group (III) (Figure 2). Pairwise comparisons of 16S rDNA sequences revealed that the native isolates within the *Sinorhizobium* group shared a similarity of 99.6% to 100% and were similarly related to their reference strains. However, two well-defined subgroups (Ia and Ib) were distinguished within the main cluster. The *S. meliloti* group (Ia) contained isolates from all three sampled *Medicago* species, grouped into four different STs, one of which is a singleton ST. The *S. medicae* group (Ib) contained four isolates grouped into a single ST, all of which were from *M. lupulina* plants collected from three different locations. The *R. leguminosarum* group (II) contained only one ST, with isolates from *M. lupulina* and *M. sativa* plants from two different plant populations. The six *R. leguminosarum* native isolates were placed in a robust lineage with their reference strains, to which 100% similarity was shared. The *Mesorhizobium* sp. group (III) contained two STs, one of which was a singleton ST. These two STs contained isolates from *M. lupulina* and *M. falcata* plants from two different plant populations. The pairwise comparison revealed that isolates within the two STs shared 98.9% similarity.

Phylogenetic analysis of the individual and concatenated *atpD*, *glnII*, and *recA* chromosomal genes was used to classify the native isolates. All the individual chromosomal markers (*atpD*, *glnII*, *recA*) followed the same clustering pattern and maintained the same four distinct groups (Ia, Ib, II, III) with varying levels of intra-group diversity (Supplementary Figures S3–S5). The native bacterial isolates grouped into several well-defined phyletic lineages, together with their most closely affiliated strains, to which they shared sequence identity values ranging from 97.2 to 100% for *atpD*, 98 to 100% for *glnII*, and 96.4 to 100% for *recA*. The *glnII* phylogeny showed greater variability, and the isolates were placed in sixteen STs (Supplementary Figure S4). Ten STs were found in the *recA* phylogeny (Supplementary Figure S5), whereas the *atpD* gene was less discriminative at strain level, with nine STs being distributed across the phylogenetic tree (Supplementary Figure S3). Although they carried highly similar 16S rDNA sequences, the isolates comprising the *Sinorhizobium* cluster revealed a more diverse genetic background: in all chromosomal phylogenies, they could be confidently grouped into two groups, defined as *S. meliloti* (Ia) and *S. medicae* (Ib). Pairwise comparisons showed that the two *Sinorhizobium* clades were distantly related to one another, with sequence identity varying from 92% to 93.9% in the described chromosomal phylogenies.

The phylogeny inferred from the concatenated sequences of the three chromosomal markers and their corresponding most similar sequences of reference strains obtained from GenBank was consistent with the individual phylogenies but resolved the native rhizobial isolates into more robust lineages (Figure 3). Eighteen STs were found in the concatenated chromosomal set, of which ten were singleton STs. The *S. meliloti* group contained ten different STs, of which four were singleton STs; the *S. medicae* group contained two STs, of which one was a singleton ST; the *R. leguminosarum* group contained three STs, of which two were singleton STs, and the *Mesorhizobium* spp. group contained three STs, all of which were singleton STs. Pairwise comparisons revealed that of the STs in the MLST (Multilocus sequence typing) phylogeny, those within the *S. meliloti*, *S. medicae*, and *R. leguminosarum* groups shared high similarity ranging from 99.3 to 99.9%. In contrast, the three isolates positioned within the *Mesorhizobium* spp. clade were more distantly related to each other, with similarities ranging from 92 to 97.1%. Isolate ML3251 was the most divergent, and it only shared 92.1 to 93.6% relatedness to the *Mesorhizobium* spp. reference strains included in the tree.

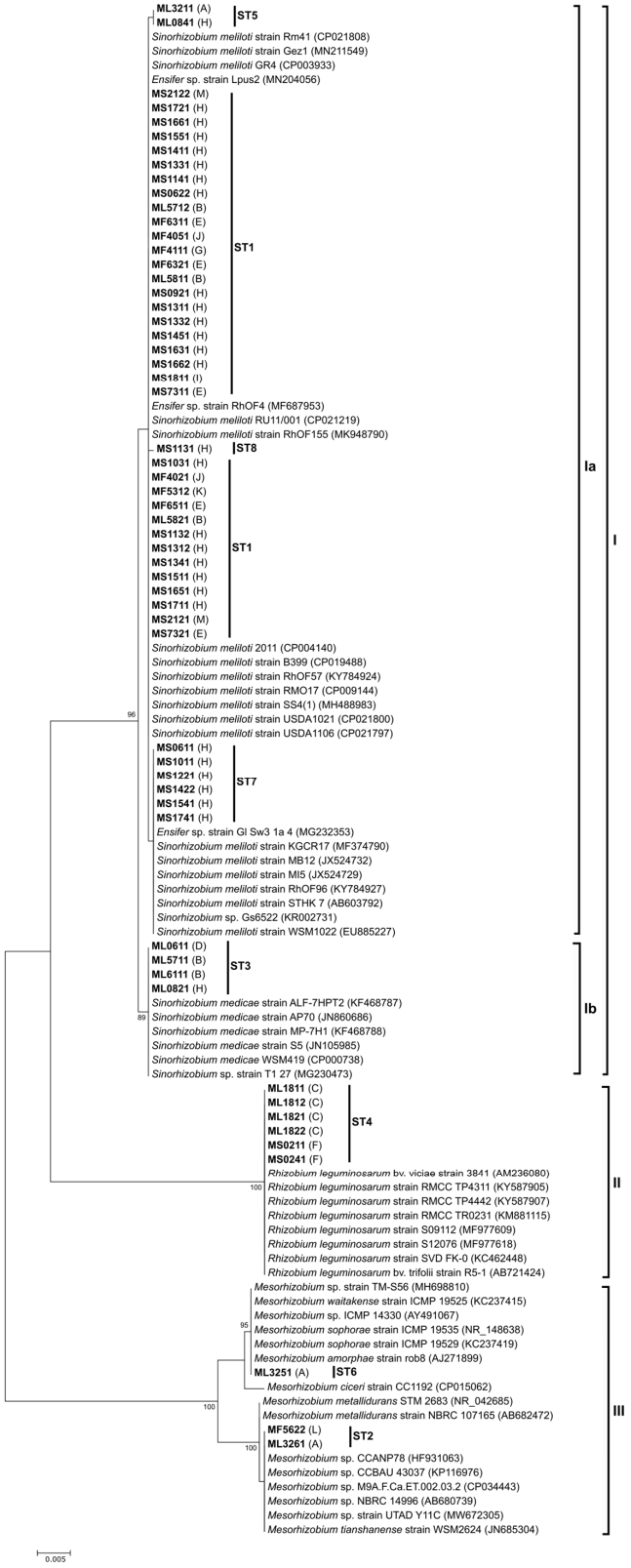


Figure 2. Maximum Likelihood tree of the 16S rDNA sequences (1345 nt); the best-fit model was found to be Jukes-Cantor and a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories). The isolates from this study are in bold, and the plant population of origin is in parentheses. The sequence types (ST) are also shown. The three main rhizobial groups are shown in Roman numerals (Ia, Ib, II, III). The 'MF', 'ML', and 'MS' in the rhizobial isolates names stand for the three *Medicago* species.

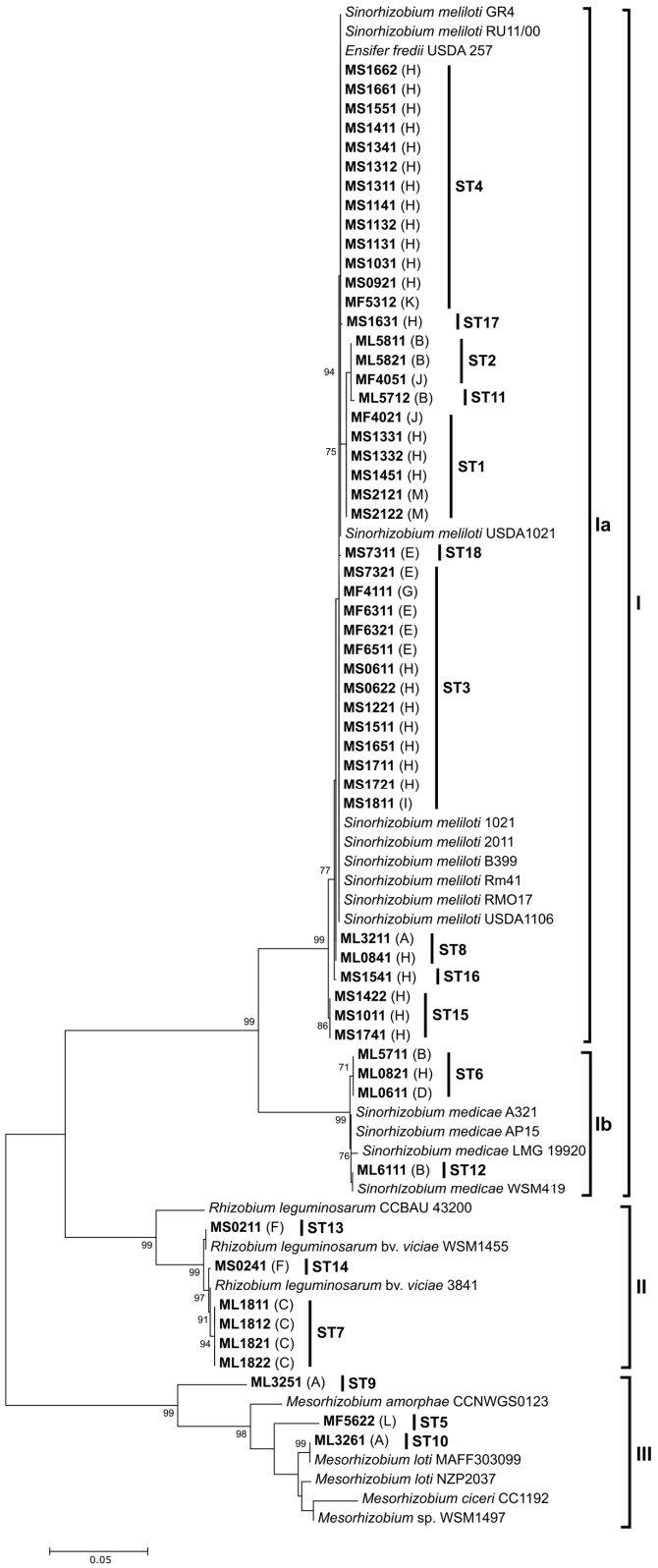


Figure 3. Maximum Likelihood tree based on the concatenated chromosomal gene sequences (*atpD* + *glnII* + *recA*, 1353 nt); the best-fit model was found to be Tamura 3-parameter and a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories). The isolates from this study are in bold, and the plant population of origin is in parentheses. The sequence types (ST) are also shown. The three main rhizobial groups are shown in Roman numerals (Ia, Ib, II, III).

The phylogenies of symbiotic genes were partially consistent with those derived from the chromosomal genes. The *nifH* phylogeny maintained the same pattern of four distinct groups and the same assignment of the isolates to the groups (Supplementary Figure S6). For *nodA*, a slightly different pattern emerged, with the MF5622, ML3251, and ML3261 isolates being clustered with the *Rhizobium leguminosarum* group and not with the *Mesorhizobium* spp. group, as was the case for the chromosomal loci (Figure 4). A pairwise comparison of the *nodA* gene alignment revealed that within the defined *Rhizobium leguminosarum* group, the native isolates were placed in two distinct, distantly related lineages, sharing only 95.2 to 96% similarity. Notably, these strains exhibited only 53.9 to 55.8% similarity to the *Mesorhizobium* spp. reference strains included in the nodA phylogram. The number of sequence types varied for each marker, from 16 for *glnII* to six for *nifH*, and the percentage of singleton sequence types was the lowest for *nifH* (16.6%) and the highest for *recA* (70%), while the rest had a percentage between 25% and 50%. The number of variable sites varied, but the ratio of variable sites (VS)/parsimony informative (PI) sites had similar values, ranging from 1 (*nodA*) to 1.1 (*atpD*). Considering the length of the sequence, 16S had the lowest percentage of parsimony informative sites (7%), while the rest of the markers had values ranging from 20% for *atpD* and *recA*, to 29% for *nodA* (Table 1).

Table 1. Sequence statistics of nucleotide polymorphisms and neutrality tests of 57 rhizobial isolates from *Medicago* species.

GM	L	ST (sST)	VarS/ParI	π	GC	Fu & Li's *D**	Fu & Li's *F**	Tajima's *D*
16S rDNA	1345	8 (2)	99/95	0.012	55.1	1.465 *	0.738	−0.752
atpD	435	9 (3)	97/88	0.044	63.8	0.547	0.261	−0.311
glnII	588	16 (8)	162/151	0.053	62.4	0.798	0.426	−0.344
recA	330	10 (7)	75/69	0.047	62.3	0.198	0.077	−0.153
nifH	168	6 (1)	47/46	0.055	60.7	1.806 **	1.231	−0.278
nodA	580	13 (5)	171/170	0.078	56.9	2.066 **	1.888 *	0.803

GM = genetic marker; L = length of sequences (in nucleotides); ST = number of sequence types; sST = number of singleton sequence types; VarS = number of variable sites; ParI = number of parsimony-informative sites; π = nucleotide diversity; GC = percent of G + C content; Fu & Li's *D**, Fu & Li's *F**, Tajima's *D* calculated using the total number of segregating sites. * Significant at $p < 0.05$, ** Significant at $p < 0.02$.

The sampled plant populations are located in four distinct regions: Rarău, Lespezi, Iași, and Oituz (Figure 1 and Supplementary Table S1). The Rarău region is located in the Northern part of the Eastern Carpathian Mountains and contains plant populations A, B, C, and D. The elevation for the sampled plants varies from ca. 500 m to just over 1000 m. Only *Medicago lupulina* plants were sampled here, and this region is the only one in this study where the rhizobial isolates belong to all of the four identified rhizobial groups. From the Lespezi region, only one plant population (E) was sampled, consisting of *M. falcata* and *M. sativa* plants. *S. meliloti* was the only rhizobial species identified and found in both plant species. The Iași region contains the F, G, H, I, and J plant populations and is the region with the lowest elevation (mean elevation less than 100 m). This is the only region where all three of the *Medicago* species were sampled; *S. meliloti* was the dominant isolate, having been found on all three *Medicago* species. The Oituz region contains the K, L, and M plant populations, with only *M. falcata* and *M. sativa* plants being sampled. The *Mesorhizobium* spp. isolate from the *M. falcata* population L (MF5622) has the same 16S sequence type as isolate ML3261 from the *M. lupulina* plant population A in the Rarău region, about 200 km away.

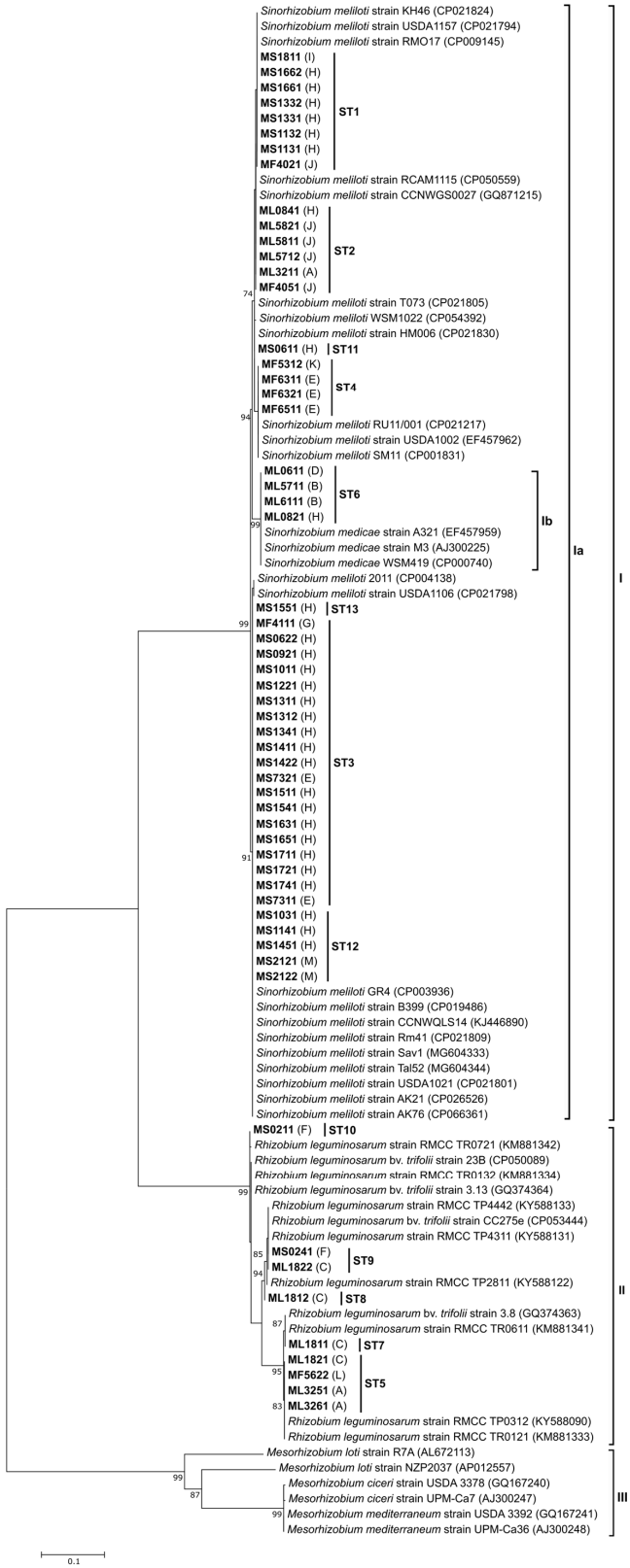
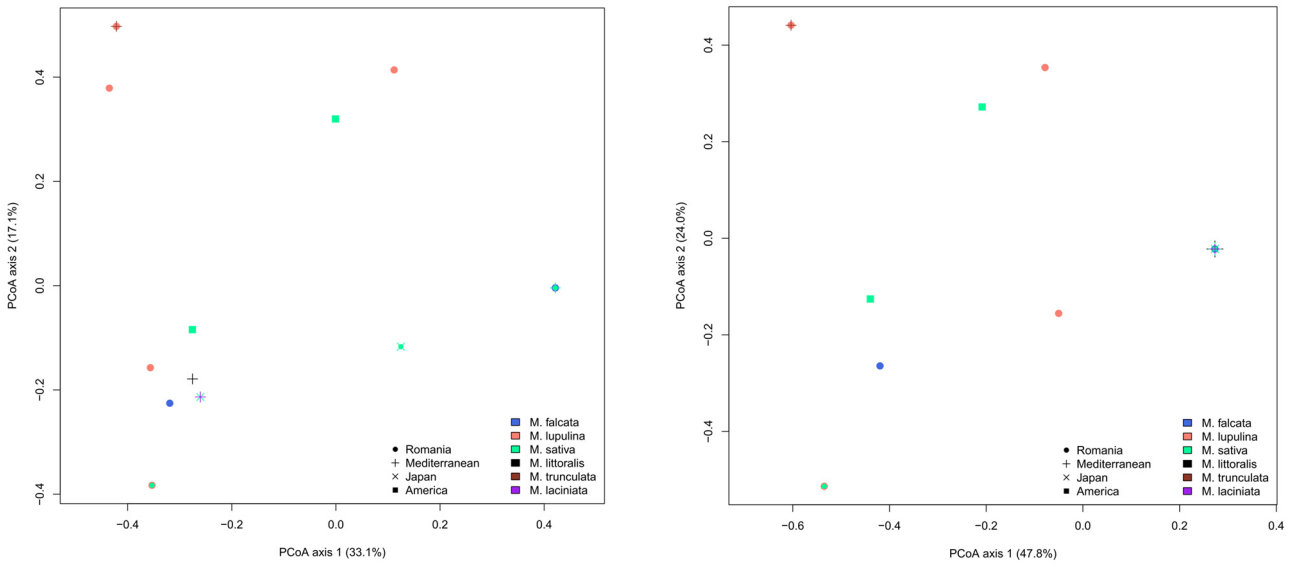


Figure 4. Maximum Likelihood tree based on the *nodA* sequences (580 nt); the best-fit model was found to be Tamura 3-parameter, and a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories). The isolates from this study are in bold, and the plant population of origin is in parentheses. The sequence types (ST) are also shown. The three main rhizobial groups are shown in Roman numerals (Ia, Ib, II).

In the present study, *S. meliloti* was found in all sampled regions, regardless of the host plant, and was identified in nine out of the fourteen plant populations and on all three of the *Medicago* species. *Sinorhizobium medicae* was identified in three plant populations (B, D, and H) from two different regions; it was identified only in *Medicago lupulina* plants. All four of the *S. medicae* isolates belonged to the same 16S rDNA sequence type. *Rhizobium leguminosarum* was found in two plant populations (C and F) from two different regions: in population C, it was identified in *M. lupulina* plants, and in population F, it was found in *M. sativa* plants. All six *R. leguminosarum* isolates had the same 16S rDNA sequence type that was previously found in *R. leguminosarum* isolates from white clover and red clover plants from the same region [51,52]. *Mesorhizobium* spp. was identified in *Medicago lupulina* in plant population A and in *M. falcata* in plant population L, and the three rhizobial isolates belonged to two distinct 16S rDNA sequence types.

3.3. Diversity of Rhizobia Associated with Medicago: Comparative Analyses

Rhizobial communities associated with *Medicago* hosts in Romania were compared with those previously observed in other geographic regions (Supplementary Tables S3 and S4) to examine whether these communities are shaped by *Medicago* species identity or by location. We found that differences in ST composition were significantly shaped by *Medicago* species identity ($F_{5,14}$ = 1.629, p = 0.011) but not by geographic region ($F_{3,14}$ = 1.229, p = 0.179; Figure 5a). This pattern arises because most commonly sampled *Medicago* species (*M. falcata*, *M. lupulina*, and *M. sativa*) share only 11–50% of STs and because ST10 is prevalent across most (61.5%) *Medicago* communities but less common in *M. lupulina* communities (20%; Table S3). Differences in species composition were not significantly affected by either *Medicago* species identity ($F_{,}$ = 1.389, p = 0.077) or geographic location ($F_{,}$ = 1.452, p = 0.071; Figure 5b).



(**a**) (**b**)

Figure 5. (**a**) PCoA plot comparing Sequence Type (ST) composition of *Medicago* spp. associated rhizobial communities using Jaccard distances. The first principal component (PCoA 1) and the second principal component (PCoA 2) explain 33.1% and 17.1% of the variance of the dataset, respectively. Distances between the points in the plot indicate the relative dissimilarity values of different regions and different *Medicago* spp. hosts. (**b**) PCoA plot comparing rhizobial species composition of *Medicago* spp., associated rhizobial communities using Jaccard distances. The first principal component (PCoA 1) and the second principal component (PCoA 2) explain 47.8% and 24.0% of the variance of the dataset, respectively. Distances between the points in the plot indicate the relative dissimilarity values of different regions and different *Medicago* spp. hosts.

4. Discussion

Building on our previous results from white clover and red clover populations [51,52], the present study adds another piece to the rhizobial diversity puzzle in Eastern Romania. Rhizobia were sampled from active root nodules of *Medicago lupulina*, *M. falcata*, and *M. sativa* plants. The initial screening and genus-level identification of rhizobia using partial 16S rDNA sequencing revealed that the rhizobial isolates belonged to four groups: *Sinorhizobium meliloti*, *S. medicae*, *Rhizobium leguminosarum*, and *Mesorhizobium* sp., and all were able to nodulate their respective host-plants in lab conditions. The 16S rDNA sequencing was sufficient to identify most isolates at the species level. Further sequencing of housekeeping and plasmid genes added sequence types and increased phylogenetic resolution among them, but the *Mesorhizobium* isolates could not be discerned at the species level.

Globally, *Medicago* species were often found to associate with *Sinorhizobium meliloti*, while other rhizobia (e.g., *Sinorhizobium medicae*) are less prevalent (Supplementary Table S3). In the present study, *Sinorhizobium meliloti* was the predominant rhizobial species, having been identified in all three *Medicago* species, regardless of elevation or soil characteristics. We identified *Sinorhizobium medicae* only in *M. lupulina* nodules. The two sinorhizobia were found nodulating both perennial and annual *Medicago* species in Mexico, with *S. meliloti* being the main symbiont of cultivated alfalfa plants [64]. In a field inoculation experiment, Roberts et al. [65] found no obvious preference among *S. meliloti* and *S. medicae* in nodulating either *M. sativa* or *M. lupulina*. In a legume survey in Tibet, Hou et al. [66] found that *S. meliloti* nodulates *M. falcata* and *M. sativa* plants, but *Rhizobium* species nodulate *M. lupulina* plants. In this study, the only sinorhizobia naturally found to form active nodules with *M. falcata* was *S. meliloti*, which has also been reported by Muntyan et al. [67]. Studies on rhizobial diversity in association with *M. lupulina* and *M. falcata* are scarce, and further sampling of natural populations of these plants could provide more information regarding their associated rhizobial community.

We found no underlying structure in the sinorhizobial isolates. The 16S rDNA ST1 of *S. meliloti* is shared among isolates from all four regions. *Sinorhizobium meliloti* isolate ML3211 from plant population A in the Rarău region shares the same 16S rDNA ST with *S. meliloti* isolate ML0841 from plant population H in the Iași region. All four *S. medicae* isolates found in plant populations B, D, and H in the two aforementioned regions also share between them the same 16S rDNA ST, showing no geographic structuring. The concatenated chromosomal set showed that the isolates from the Iași region shared identical STs with isolates from all the other three regions, but the other three regions did not share a common ST between them. The inability to discern structure could also occur because few plant populations were sampled. In a genotyping-by-sequencing study, Harrison et al. [68] similarly found no evidence of population structure within either *S. meliloti* or *S. medicae* in nodules of *M. lupulina* plants along a latitude gradient.

In Romanian *Medicago* communities, we also detected *R. leguminosarum* and two *Mesorhizobium* species. Both *R. leguminosarum* and *Mesorhizobium* spp. had previously been detected in *M. lupulina* by De Meyer et al. [40]. Consistent with the previous study in Belgium [40], which found that *Medicago lupulina* hosted the most diverse rhizobial community among all sampled *Medicago* species, we found that *M. lupulina* was the only plant species that harbored all four groups of rhizobial isolates that we identified.

Isolates found in *M. lupulina* also occurred in other host species. For example, *R. leguminosarum* bacteria harboring the same 16S rDNA sequence type that was identified in *M. lupulina* nodules in plant population C in the Rarău region are widespread in Eastern Romania, having been identified in three regions on at least four plant species, including *M. sativa* (population F from the Iaşi region), white clover (*Trifolium repens*), and red clover (*Trifolium pratense*) in multiple populations from both the Rarău and Oituz regions [51,52]. Three species of *Rhizobium* and *S. meliloti* were also identified as nodulating alfalfa varieties in China [23].

The mesorhizobia we identified in Romania belongs to two different 16S rDNA STs on well-supported clades. Both sequence types have been observed elsewhere in the world and are able to modulate a diversity of legume species. Isolate ML3251 shares a sequence type with mesorhizobia identified on *Sophora microphylla* from New Zealand [69], *Oxytropis* sp. from Canada [70], and *Robinia pseudoacacia* from South Korea [71] and Germany [72]. Isolates ML3261 and MF5622 share the same 16S rDNA ST with mesorhizobia from *Cicer arietinum* in Ethiopia [73], *Glycine max* in Latvia (unpublished, GenBank accession number OQ236321.1, https://www.ncbi.nlm.nih.gov/nuccore/OQ236321, accessed on 13 July 2023), *Cicer canariense* in the Canary Islands [74], *Lotononis* sp. in South Africa [75], and *Lotus corniculatus* in the USA [76]. *Mesorhizobium* species have previously been sampled from *M. lupulina*. For example, Zeng et al. [77] used 16S rDNA sequencing to identify two species of *Mesorhizobium* nodulating *M. lupulina* plants in a karstic region of China. To our knowledge, we also provide the first report of *Mesorhizobium* species in *M. falcata* nodules.

The two 16S rDNA mesorhizobia clades from this study are congruent with clades delineated by the other sequenced genetic markers, except *nodA*. The *nodA* sequences from the isolated *Mesorhizobium* species cluster together with the *nodA* sequences from *Rhizobium leguminosarum* isolates and are closely related to the *nodA* STs previously identified in red clover and white clover plants [51,52]. This phylogenetic incongruence could result from the horizontal transfer of symbiotic genes between rhizobia, a geographically widespread and common occurrence that is unrestricted between and within rhizobial genera [78]. Previous studies have detected the transfer of *nodA* genes from sinorhizobial species to mesorhizobia. Gerding et al. [79] provided evidence for lateral gene transfer in a *Mesorhizobium* strain nodulating *Lessertia* in South Africa. That strain harbored a *nodA* gene closely related to the *nodA* that we found in *S. meliloti*. Fterich et al. [80] identified a *Mesorhizobium* isolate from *Prosopis farcta* in Tunisia containing a *nodA* sequence identical to the one we found in *S. medicae*. This previous evidence, coupled with our own findings, could indicate that mesorhizobia are especially susceptible to the acquisition of foreign nodulation genes capable of establishing symbioses in specific soil conditions or under the constraint of a more selective plant species. Epstein and Tiffin [81] showed that the genes involved in nodule formation display rates of transfer 50% or higher than the nitrogen fixation genes. Our results could also explain why the *nifH* sequences of mesorhizobia from his study cluster with *nifH* sequences of other *Mesorhizobium* species, but the *nodA* sequences cluster outside of *Mesorhizobium*.

The 16S rDNA ST comparative analysis results, indicated that various *Medicago* species generally share the same rhizobial species across the globe. However, *Mesorhizobium* and *R. leguminosarum*, which were identified in this study, had not been reported before. Moreover, it is possible that different *Medicago* species prefer different STs. This may provide an explanation for contrasting results seen in different studies, where apparent species preferences are evident in some cases (e.g., here, [64,66]) while absent in others (e.g., [65]). It is unclear whether *Medicago* spp. have preferences for certain strains regardless of species identity or whether this is the result of a lack of studies on the diversity of rhizobia associated with *Medicago*. Clearly, more extensive sampling is needed to test this preference hypothesis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14092156/s1, Figure S1: Cluster analysis of phenotypic features by UPGMA; Figure S2: Basic descriptive statistics of inoculation experiments; Figure S3: Maximum Likelihood tree of the atpD sequences; Figure S4: Maximum Likelihood tree of the glnII sequences; Figure S5: Maximum Likelihood tree of the recA sequences; Figure S6: Maximum Likelihood tree of the nifH sequences; Table S1: Sampled Medicago populations, physical characteristics of sampling sites and rhizobial isolates used in the present study; Table S2: Phenotypic characterization of rhizobial isolates associated with Medicago plants and clusters defined by numerical taxonomy; Table S3: Global distribution of STs in *Medicago* spp.; Table S4: List of sequences used in the phylogenetic analysis.

Author Contributions: A.S.: Conceptualization, Investigation, Formal analysis, Visualization, Writing—original draft; J.V.C.: Conceptualization, Formal analysis, Writing—original draft; C.M.R.: Investigation, Formal analysis, Writing—original draft; C.S.: Investigation, Formal analysis; C.C.: Formal analysis, Writing—original draft; E.L.S.: Resources, Writing—review and editing; C.I.: Resources; D.T.: Resources, Writing—review and editing; E.F.: Conceptualization, Writing—review and editing; R.C.E.: Conceptualization, Investigation, Formal analysis, Writing—original draft, Resources, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the UEFISCDI PN-II-ID-PCE-2011-3-1011 funding grant (contract no: 292/5.10.2011), the Core-Program, within the National Plan for Research, Development and Innovation 2022-2027 (project no. 7N/23020402/2023) and the project ResPonSE, (contract no. 760010/30.12.2022), developed with the support of the Romanian Ministry of Research, Innovation and Digitalization. This work was supported by NSF grants DEB-1457508 and IOS-1759048 awarded to Ellen Simms, which supported J.V.C. and C.C. J.V.C. was also supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy–EXC 2051–Project-ID 390713860 and by the European Research Council (ERC) Consolidator grant 865694: DiversiPHI, the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy–EXC 2051–Project-ID 390713860, and the Alexander von Humboldt Foundation in the context of an Alexander von Humboldt-Professorship founded by German Federal Ministry of Education and Research.

Data Availability Statement: DNA sequences are deposited in GenBank and are publicly available. All data supporting the findings of this study are available in the paper and its Supplementary Information section.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Masson-Boivin, C.; Giraud, E.; Perret, X.; Batut, J. Establishing nitrogen-fixing symbiosis with legumes: How many rhizobium recipes? *Trends Microbiol.* **2009**, *17*, 458–466. [CrossRef]
2. Wang, D.; Yang, S.; Tang, F.; Zhu, H. Symbiosis Specificity in the Legume: Rhizobial Mutualism. *Cell. Microbiol.* **2012**, *14*, 334–342. [CrossRef] [PubMed]
3. Wang, Q.; Liu, J.; Zhu, H. Genetic and Molecular Mechanisms Underlying Symbiotic Specificity in Legume-Rhizobium Interactions. *Front. Plant Sci.* **2018**, *9*, 313. [CrossRef] [PubMed]
4. Lindström, K.; Mousavi, S.A. Effectiveness of nitrogen fixation in rhizobia. *Microb. Biotechnol.* **2019**, *13*, 1314–1335. [CrossRef]
5. Schulte, C.C.M.; Borah, K.; Wheatley, R.M.; Terpolilli, J.J.; Saalbach, G.; Crang, N.; de Groot, D.H.; Ratcliffe, R.G.; Kruger, N.J.; Papachristodoulou, A.; et al. Metabolic control of nitrogen fixation in rhizobium-legume symbioses. *Sci. Adv.* **2021**, *7*, eabh2433. [CrossRef] [PubMed]
6. Masson-Boivin, C.; Sachs, J.L. Symbiotic nitrogen fixation by rhizobia—The roots of a success story. *Curr. Opin. Plant Biol.* **2018**, *44*, 7–15. [CrossRef]
7. Herridge, D.F.; Peoples, M.B.; Boddey, R.M. Global inputs of biological nitrogen fixation in agricultural systems. *Plant Soil* **2008**, *311*, 1–18. [CrossRef]
8. Hénault, C.; Barbier, E.; Hartmann, A.; Revellin, C. New Insights into the Use of Rhizobia to Mitigate Soil N_2O Emissions. *Agriculture* **2022**, *12*, 271. [CrossRef]
9. Li, J.; Wang, J.; Liu, H.; Macdonald, C.A.; Singh, B.K. Application of microbial inoculants significantly enhances crop productivity: A meta-analysis of studies from 2010 to 2020. *J. Sustain. Agric. Environ.* **2022**, *1*, 216–225. [CrossRef]
10. Maitra, S.; Brestic, M.; Bhadra, P.; Shankar, T.; Praharaj, S.; Palai, J.B.; Shah, M.M.R.; Barek, V.; Ondrisik, P.; Skalický, M.; et al. Bioinoculants—Natural Biological Resources for Sustainable Plant Production. *Microorganisms* **2021**, *10*, 51. [CrossRef]
11. Chamkhi, I.; Cheto, S.; Geistlinger, J.; Zeroual, Y.; Kouisni, L.; Bargaz, A.; Ghoulam, C. Legume-based intercropping systems promote beneficial rhizobacterial community and crop yield under stressing conditions. *Ind. Crop. Prod.* **2022**, *183*, 114958. [CrossRef]
12. Kebede, E. Contribution, Utilization, and Improvement of Legumes-Driven Biological Nitrogen Fixation in Agricultural Systems. *Front. Sustain. Food Syst.* **2021**, *5*, 767998. [CrossRef]
13. Layek, J.; Das, A.; Mitran, T.; Nath, C.; Meena, R.S.; Yadav, G.S.; Shivakumar, B.G.; Kumar, S.; Lal, R. Cereal+Legume Intercropping: An Option for Improving Productivity and Sustaining Soil Health. In *Legumes for Soil Health and Sustainable Management*; Meena, R.S., Das, A., Yadav, G.S., Lal, R., Eds.; Springer: Singapore, 2018; pp. 347–386, ISBN 9789811302534. [CrossRef]
14. Fageria, N.K. Green Manuring in Crop Production. *J. Plant Nutr.* **2007**, *30*, 691–719. [CrossRef]
15. Gatsios, A.; Ntatsi, G.; Celi, L.; Said-Pullicino, D.; Tampakaki, A.; Savvas, D. Legume-Based Mobile Green Manure Can Increase Soil Nitrogen Availability and Yield of Organic Greenhouse Tomatoes. *Plants* **2021**, *10*, 2419. [CrossRef] [PubMed]

16. Meena, B.L.; Fagodiya, R.K.; Prajapat, K.; Dotaniya, M.L.; Kaledhonkar, M.J.; Sharma, P.C.; Meena, R.S.; Mitran, T.; Kumar, S. Legume Green Manuring: An Option for Soil Sustainability. In *Legumes for Soil Health and Sustainable Management*; Meena, R.S., Das, A., Yadav, G.S., Lal, R., Eds.; Springer: Singapore, 2018; pp. 387–408, ISBN 9789811302534. [CrossRef]
17. Lindström, K.; Murwira, M.; Willems, A.; Altier, N. The biodiversity of beneficial microbe-host mutualism: The case of rhizobia. *Res. Microbiol.* **2010**, *161*, 453–463. [CrossRef]
18. Argaw, A.; Tsigie, A. Indigenous rhizobia population influences the effectiveness of *Rhizobium* inoculation and need of inorganic N for common bean (*Phaseolus vulgaris* L.) production in eastern Ethiopia. *Chem. Biol. Technol. Agric.* **2015**, *2*, 19. [CrossRef]
19. Athul, P.P.; Patra, R.K.; Sethi, D.; Panda, N.; Mukhi, S.K.; Padhan, K.; Sahoo, S.K.; Sahoo, T.R.; Mangaraj, S.; Pradhan, S.R.; et al. Efficient native strains of rhizobia improved nodulation and productivity of French bean (*Phaseolus vulgaris* L.) under rainfed condition. *Front. Plant Sci.* **2022**, *13*, 1048696. [CrossRef]
20. Castellano-Hinojosa, A.; Mora, C.; Strauss, S.L. Native Rhizobia Improve Plant Growth, Fix N_2, and Reduce Greenhouse Emissions of Sunnhemp More than Commercial Rhizobia Inoculants in Florida Citrus Orchards. *Plants* **2022**, *11*, 3011. [CrossRef] [PubMed]
21. Koskey, G.; Mburu, S.W.; Njeru, E.M.; Kimiti, J.M.; Ombori, O.; Maingi, J.M. Potential of Native Rhizobia in Enhancing Nitrogen Fixation and Yields of Climbing Beans (*Phaseolus vulgaris* L.) in Contrasting Environments of Eastern Kenya. *Front. Plant Sci.* **2017**, *8*, 443. [CrossRef]
22. Nguyen, T.T.; Atieno, M.; Herrmann, L.; Nakasathien, S.; Sarobol, E.; Wongkaew, A.; Nguyen, K.T.; Lesueur, D. Does inoculation with native rhizobia enhance nitrogen fixation and yield of cowpea through legume-based intercropping in the northern mountainous areas of Vietnam? *Exp. Agric.* **2020**, *56*, 825–836. [CrossRef]
23. Kang, W.; Shi, S.; Xu, L. Diversity and symbiotic divergence of endophytic and non-endophytic rhizobia of Medicago sativa. *Ann. Microbiol.* **2018**, *68*, 247–260. [CrossRef]
24. van Berkum, P.; Elia, P.; Eardly, B.D. Multilocus Sequence Typing as an Approach for Population Analysis of *Medicago*-Nodulating Rhizobia. *J. Bacteriol.* **2006**, *188*, 5570–5577. [CrossRef]
25. van Berkum, P.; Badri, Y.; Elia, P.; Aouani, M.E.; Eardly, B.D. Chromosomal and Symbiotic Relationships of Rhizobia Nodulating *Medicago truncatula* and *M. laciniata*. *Appl. Environ. Microbiol.* **2007**, *73*, 7597–7604. [CrossRef]
26. Lemaire, B.; Dlodlo, O.; Chimphango, S.; Stirton, C.; Schrire, B.; Boatwright, J.S.; Honnay, O.; Smets, E.; Sprent, J.; James, E.K.; et al. Symbiotic diversity, specificity and distribution of rhizobia in native legumes of the Core Cape Subregion (South Africa). *FEMS Microbiol. Ecol.* **2015**, *91*, 1–17. [CrossRef]
27. Van Cauwenberghe, J.; Verstraete, B.; Lemaire, B.; Lievens, B.; Michiels, J.; Honnay, O. Population structure of root nodulating *Rhizobium leguminosarum* in *Vicia cracca* populations at local to regional geographic scales. *Syst. Appl. Microbiol.* **2014**, *37*, 613–621. [CrossRef] [PubMed]
28. Kozieł, M.; Kalita, M.; Janczarek, M. Genetic diversity of microsymbionts nodulating *Trifolium pratense* in subpolar and temperate climate regions. *Sci. Rep.* **2022**, *12*, 12144. [CrossRef]
29. Bessadok, K.; Navarro-Torre, S.; Fterich, A.; Caviedes, M.A.; Pajuelo, E.; Rodríguez-Llorente, I.D.; Mars, M. Diversity of rhizobia isolated from Tunisian arid soils capable of forming nitrogen-fixing symbiosis with *Anthyllis henoniana*. *J. Arid. Environ.* **2021**, *188*, 104467. [CrossRef]
30. Efstathiadou, E.; Ntatsi, G.; Savvas, D.; Tampakaki, A.P. Genetic characterization at the species and symbiovar level of indigenous rhizobial isolates nodulating *Phaseolus vulgaris* in Greece. *Sci. Rep.* **2021**, *11*, 8674. [CrossRef]
31. Tampakaki, A.P.; Fotiadis, C.T.; Ntatsi, G.; Savvas, D. Phylogenetic multilocus sequence analysis of indigenous slow-growing rhizobia nodulating cowpea (*Vigna unguiculata* L.) in Greece. *Syst. Appl. Microbiol.* **2017**, *40*, 179–189. [CrossRef]
32. Simbine, M.G.; Mohammed, M.; Jaiswal, S.K.; Dakora, F.D. Functional and genetic diversity of native rhizobial isolates nodulating cowpea (*Vigna unguiculata* L. Walp.) in Mozambican soils. *Sci. Rep.* **2021**, *11*, 12747. [CrossRef]
33. Steele, K.P.; Ickert-Bond, S.M.; Zarre, S.; Wojciechowski, M.F. Phylogeny and character evolution in *Medicago* (Leguminosae): Evidence from analyses of plastid trnK/matK and nuclear GA3ox1 sequences. *Am. J. Bot.* **2010**, *97*, 1142–1155. [CrossRef]
34. Kang, Y.; Han, Y.; Torres-Jerez, I.; Wang, M.; Tang, Y.; Monteros, M.; Udvardi, M. System responses to long-term drought and re-watering of two contrasting alfalfa varieties. *Plant J.* **2011**, *68*, 871–889. [CrossRef]
35. Zhang, L.-L.; Zhao, M.-G.; Tian, Q.-Y.; Zhang, W.-H. Comparative studies on tolerance of Medicago truncatula and Medicago falcata to freezing. *Planta* **2011**, *234*, 445–457. [CrossRef]
36. Wang, H.; Coulman, B.; Bai, Y.; Tar'an, B.; Biligetu, B. Genetic diversity and local adaption of alfalfa populations (*Medicago sativa* L.) under long-term grazing. *Sci. Rep.* **2023**, *13*, 1632. [CrossRef]
37. Epstein, B.; Branca, A.; Mudge, J.; Bharti, A.K.; Briskine, R.; Farmer, A.D.; Sugawara, M.; Young, N.D.; Sadowsky, M.J.; Tiffin, P. Population Genomics of the Facultatively Mutualistic Bacteria *Sinorhizobium meliloti* and S. *medicae*. *PLoS Genet.* **2012**, *8*, e1002868. [CrossRef]
38. Rome, S.; Brunel, B.; Normand, P.; Fernandez, M.; Cleyet-Marel, J.C. Evidence that two genomic species of Rhizobium are associated with Medicago truncatula. *Arch. Microbiol.* **1996**, *165*, 285–288. [CrossRef]
39. Bromfield, E.S.P.; Tambong, J.T.; Cloutier, S.; Prévost, D.; Laguerre, G.; van Berkum, P.; Thi, T.V.T.; Assabgui, R.; Barran, L.R. *Ensifer*, *Phyllobacterium* and *Rhizobium* species occupy nodules of *Medicago sativa* (alfalfa) and *Melilotus alba* (sweet clover) grown at a Canadian site without a history of cultivation. *Microbiology* **2010**, *156*, 505–520. [CrossRef]

40. De Meyer, S.E.; Van Hoorde, K.; Vekeman, B.; Braeckman, T.; Willems, A. Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium). *Soil Biol. Biochem.* **2011**, *43*, 2384–2396. [CrossRef]
41. Ciocârlan, V. *Flora Ilustrată a României: Pteridophyta et Spermatophyta (The Illustrated Flora of Romania)*, 2nd ed.; Ceres: Bucharest, Romania, 2000; ISBN 973-40-0495-6. (In Romanian)
42. Vîrdol, D.C. (Ed.) *The Romanian Statistical Yearbook 2022*; National Institute of Statistics (NIS): Bucharest, Romania, 2023; pp. 476–477, ISBN 1220-3246. Available online: https://insse.ro/cms/en/content/romanian-statistical-yearbook-book-format-5 (accessed on 11 October 2023).
43. Beattie, G.A.; Handelsman, J. A rapid method for the isolation and identification of *Rhizobium* from root nodules. *J. Microbiol. Methods* **1989**, *9*, 29–33. [CrossRef]
44. Vincent, J.M. *A Manual for the Practical Study of the Root-Nodule Bacteria (IBP Handbuch No. 15 des International Biology Program, London)*; Blackwell Scientific Publishing: Oxford, UK; Edinburgh, UK, 1970. [CrossRef]
45. Somasegaran, P.; Hoben, H.J. Quantifying the Growth of Rhizobia. In *Handbook for Rhizobia: Methods in Legume-Rhizobium Technology*; Somasegaran, P., Hoben, H.J., Eds.; Springer: New York, NY, USA, 1994; pp. 47–57, ISBN 978-1-4613-8375-8. [CrossRef]
46. Frioni, L.; Rodríguez, A.; Meerhoff, M. Differentiation of rhizobia isolated from native legume trees in Uruguay. *Appl. Soil Ecol.* **2001**, *16*, 275–282. [CrossRef]
47. Gao, J.-L.; Turner, S.L.; Kan, F.L.; Wang, E.T.; Tan, Z.Y.; Qiu, Y.H.; Gu, J.; Terefework, Z.; Young, J.P.W.; Lindström, K.; et al. *Mesorhizobium septentrionale* sp. nov. and *Mesorhizobium temperatum* sp. nov., isolated from *Astragalus adsurgens* growing in the northern regions of China. *Int. J. Syst. Evol. Microbiol.* **2004**, *54*, 2003–2012. [CrossRef] [PubMed]
48. Josey, D.P.; Beynon, J.L.; Johnston, A.W.B.; Beringer, J.E. Strain Identification in *Rhizobium* Using Intrinsic Antibiotic Resistance. *J. Appl. Bacteriol.* **1979**, *46*, 343–350. [CrossRef]
49. Mpepereki, S.; Makonese, F.; Wollum, A.G. Physiological characterization of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils. *Symbiosis* **1997**, *22*, 275–292.
50. Sneath, P.H.A.; Sokal, R.R. *Numerical Taxonomy: The Principles and Practice of Numerical Classification*; W. H. Freeman: New York, NY, USA, 1973; ISBN 978-0-7167-0697-7.
51. Efrose, R.C.; Rosu, C.M.; Stedel, C.; Stefan, A.; Sirbu, C.; Gorgan, L.D.; Labrou, N.E.; Flemetakis, E. Molecular diversity and phylogeny of indigenous *Rhizobium leguminosarum* strains associated with *Trifolium repens* plants in Romania. *Antonie Van Leeuwenhoek* **2017**, *111*, 135–153. [CrossRef]
52. Stefan, A.; Van Cauwenberghe, J.; Rosu, C.M.; Stedel, C.; Labrou, N.E.; Flemetakis, E.; Efrose, R.C. Genetic diversity and structure of *Rhizobium leguminosarum* populations associated with clover plants are influenced by local environmental variables. *Syst. Appl. Microbiol.* **2018**, *41*, 251–259. [CrossRef]
53. Rozas, J.; Ferrer-Mata, A.; Sánchez-DelBarrio, J.C.; Guirao-Rico, S.; Librado, P.; Ramos-Onsins, S.E.; Sánchez-Gracia, A. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Mol. Biol. Evol.* **2017**, *34*, 3299–3302. [CrossRef] [PubMed]
54. Sayers, E.W.; Cavanaugh, M.; Clark, K.; Pruitt, K.D.; Schoch, C.L.; Sherry, S.T.; Karsch-Mizrachi, I. GenBank. *Nucleic Acids Res.* **2020**, *49*, D92–D96. [CrossRef] [PubMed]
55. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic Local Alignment Search Tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
56. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]
57. Goslee, S.C.; Urban, D.L. The ecodist Package for Dissimilarity-based Analysis of Ecological Data. *J. Stat. Softw.* **2007**, *22*, 1–19. [CrossRef]
58. Oksanen, J.; Simpson, G.L.; Blanchet, F.G.; Kindt, R.; Legendre, P.; Minchin, P.R.; O'Hara, R.B.; Solymos, P.; Stevens, M.H.H.; Szoecs, E.; et al. Vegan: Community Ecology Package 2022. Available online: https://cran.r-project.org/web/packages/vegan/index.html (accessed on 11 October 2023).
59. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2020; Available online: https://www.r-project.org/ (accessed on 15 May 2023).
60. Dray, S.; Dufour, A.-B. The ade4 Package: Implementing the Duality Diagram for Ecologists. *J. Stat. Softw.* **2007**, *22*, 1–20. [CrossRef]
61. Siberchicot, A.; Julien-Laferrière, A.; Dufour, A.-B.; Thioulouse, J.; Dray, S. adegraphics: An S4 Lattice-Based Package for the Representation of Multivariate Data. *R J.* **2017**, *9*, 198–212. [CrossRef]
62. QGIS Development Team. 2023. Available online: https://www.qgis.org/ (accessed on 15 May 2023).
63. Inkscape Project, 2020. Available online: https://inkscape.org/ (accessed on 15 May 2023).
64. Silva, C.; Kan, F.L.; Martínez-Romero, E. Population genetic structure of *Sinorhizobium meliloti* and *S. medicae* isolated from nodules of *Medicago* spp. in Mexico. *FEMS Microbiol. Ecol.* **2007**, *60*, 477–489. [CrossRef]
65. Roberts, R.; Jackson, R.W.; Mauchline, T.H.; Hirsch, P.R.; Shaw, L.J.; Döring, T.F.; Jones, H.E. Is there sufficient *Ensifer* and *Rhizobium* species diversity in UK farmland soils to support red clover (*Trifolium pratense*), white clover (*T. repens*), lucerne (*Medicago sativa*) and black medic (*M. lupulina*)? *Appl. Soil Ecol.* **2017**, *120*, 35–43. [CrossRef]
66. Hou, B.C.; Wang, E.T.; Li, Y.; Jia, R.Z.; Chen, W.F.; Man, C.X.; Sui, X.H.; Chen, W.X. Rhizobial Resource Associated with Epidemic Legumes in Tibet. *Microb. Ecol.* **2009**, *57*, 69–81. [CrossRef]

67. Muntyan, V.S.; Baturina, O.A.; Afonin, A.M.; Cherkasova, M.E.; Laktionov, Y.V.; Saksaganskaya, A.S.; Kabilov, M.R.; Roumiantseva, M.L. Draft Genome Sequence of *Sinorhizobium meliloti* AK555. *Microbiol. Resour. Announc.* **2019**, *8*, e01567-18. [CrossRef]
68. Harrison, T.L.; Wood, C.W.; Heath, K.D.; Stinchcombe, J.R. Geographically Structured Genetic Variation in the *Medicago lupulina-Ensifer* Mutualism. *Evolution* **2017**, *71*, 1787–1801. [CrossRef]
69. De Meyer, S.E.; Tan, H.W.; Andrews, M.; Heenan, P.B.; Willems, A. *Mesorhizobium calcicola* sp. nov., *Mesorhizobium waitakense* sp. nov., *Mesorhizobium sophorae* sp. nov., *Mesorhizobium newzealandense* sp. nov. and *Mesorhizobium kowhaii* sp. nov. isolated from *Sophora* root nodules. *Int. J. Syst. Evol. Microbiol.* **2016**, *66*, 786–795. [CrossRef] [PubMed]
70. Duan, Y.F.; Grogan, P.; Walker, V.K.; diCenzo, G.C. Whole genome sequencing of mesorhizobia isolated from northern Canada. *Can. J. Microbiol.* **2022**, *68*, 661–673. [CrossRef]
71. Kang, J.W.; Song, J.; Doty, S.L.; Lee, D.K. Diversity of Rhizobia Associated with Leguminous Trees Growing in South Korea. *J. Basic Microbiol.* **2013**, *53*, 291–298. [CrossRef]
72. Ulrich, A.; Zaspel, I. Phylogenetic diversity of rhizobial strains nodulating *Robinia pseudoacacia* L. *Microbiology* **2000**, *146 Pt 11*, 2997–3005. [CrossRef] [PubMed]
73. Greenlon, A.; Chang, P.L.; Damtew, Z.M.; Muleta, A.; Carrasquilla-Garcia, N.; Kim, D.; Nguyen, H.P.; Suryawanshi, V.; Krieg, C.P.; Yadav, S.K.; et al. Global-level population genomics reveals differential effects of geography and phylogeny on horizontal gene transfer in soil bacteria. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 15200–15209. [CrossRef] [PubMed]
74. Armas-Capote, N.; Pérez-Yépez, J.; Martínez-Hidalgo, P.; Garzón-Machado, V.; Del Arco-Aguilar, M.; Velázquez, E.; León-Barrios, M. Core and symbiotic genes reveal nine *Mesorhizobium* genospecies and three symbiotic lineages among the rhizobia nodulating *Cicer canariense* in its natural habitat (La Palma, Canary Islands). *Syst. Appl. Microbiol.* **2014**, *37*, 140–148. [CrossRef]
75. Ardley, J.K.; Reeve, W.G.; O'Hara, G.W.; Yates, R.J.; Dilworth, M.J.; Howieson, J.G. Nodule morphology, symbiotic specificity and association with unusual rhizobia are distinguishing features of the genus *Listia* within the Southern African crotalarioid clade *Lotononis s.l. Ann. Bot.* **2013**, *112*, 1–15. [CrossRef]
76. Qian, J.; Parker, M.A. Contrasting nifD and Ribosomal Gene Relationships Among *Mesorhizobium* from *Lotus oroboides* in Northern Mexico. *Syst. Appl. Microbiol.* **2002**, *25*, 68–73. [CrossRef] [PubMed]
77. Zeng, Q.; Wei, X.; Wei, X.; Ou, E.; Ji, Y.; Shu, J.; Long, Z. Research on Resource Exploration, Nitrogen Fixation Characteristics and Diversity of Rhizobia of *Medicago lupulina* in Karst Mountainous Area of Guizhou. *Acta Agrestia Sin.* **2022**, *30*, 1891–1899. [CrossRef]
78. Andrews, M.; De Meyer, S.; James, E.K.; Stępkowski, T.; Hodge, S.; Simon, M.F.; Young, J.P.W. Horizontal Transfer of Symbiosis Genes within and between Rhizobial Genera: Occurrence and Importance. *Genes* **2018**, *9*, 321. [CrossRef]
79. Gerding, M.; O'Hara, G.W.; Bräu, L.; Nandasena, K.; Howieson, J.G. Diverse *Mesorhizobium* spp. with unique nodA nodulating the South African legume species of the genus *Lessertia*. *Plant Soil* **2012**, *358*, 385–401. [CrossRef]
80. Fterich, A.; Mahdhi, M.; Caviedes, M.A.; Pajuelo, E.; Rivas, R.; Rodriguez-Llorente, I.D.; Mars, M. Characterization of root-nodulating bacteria associated to *Prosopis farcta* growing in the arid regions of Tunisia. *Arch. Microbiol.* **2011**, *193*, 385–397. [CrossRef]
81. Epstein, B.; Tiffin, P. Comparative genomics reveals high rates of horizontal transfer and strong purifying selection on rhizobial symbiosis genes. *Proc. Biol. Sci.* **2021**, *288*, 20201804. [CrossRef]

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