1 **Evolution and taxonomy of native mesorhizobia nodulating medicinal *Glycyrrhiza* species in**
2 **China**

3 Seyed Abdollah Mousavi^{1*†}, Li Li^{2,3†}, Gehong Wei⁴, Leena Räsänen², Kristina Lindström¹

4 ¹ University of Helsinki, Department of Environmental Sciences, Helsinki, Finland.

5
6 ² University of Helsinki, Department of Food and Environmental Sciences, Helsinki, Finland.

7
8 ³ Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and
9 Geography, Chinese Academy of Sciences, Urumqi, 830011, China.

10
11 ⁴ Northwest A&F University, College of Life Sciences, Yangling, Shaanxi, China.

12
13
14 *Corresponding author. email address: s.abdollah.mousavi@gmail.com or
15 seyed.mousavi@helsinki.fi (Seyed Abdollah MOUSAVI).

16 †The authors wish it to be known that, in their opinion, the first two authors should be regarded as
17 Joint First Authors.

18 Department of Environmental Sciences, Viikinkaari 2a, P.O. Box 65, University of Helsinki, Helsinki,
19 Finland, FIN-00014.

20
21 **Running title:** Phylogeny of mesorhizobia nodulating *Glycyrrhiza*

22
23 **Abstract**

24 Previously, 159 bacterial strains were isolated from the root nodules of wild perennial *Glycyrrhiza*
25 legume species grown on 40 sites in central and north-western China, in which 57 strains were
26 classified as "true symbionts" belonging to the genus *Mesorhizobium* based on amplified fragment
27 length polymorphism (AFLP) genomic fingerprinting and partial sequences of the 16S rRNA gene
28 [20]. In the present work, the phylogeny of *Glycyrrhiza* nodulating mesorhizobia was further
29 examined by multilocus sequence analysis (MLSA). The concatenated gene tree of three
30 housekeeping genes (16S rRNA, *recA*, and *rpoB*) of 59 strains including the 29 mesorhizobial test
31 strains and 30 type mesorhizobial species, was constructed applying the maximum likelihood method
32 and Bayesian inference. In the concatenated gene tree, the 29 test strains were distributed in seven
33 separate clades. Seventeen test strains clustered with *M. tianshanense*, *M. temperatum*, *M. muleiense*,
34 and *M. alhagi* with high bootstrap support (BS>85%). Eight test strains did not cluster with any of
35 the described *Mesorhizobium* species. Based on the results, we proposed these eight test strains might

belong to a putative new species of the genus *Mesorhizobium*. The sequences of three accessory genes (*nodA*, *nodC*, and *nifH*) of the test strains were also analysed and were compared with those of representatives of the 30 described mesorhizobial species. The results showed that mesorhizobia involved in symbiosis with *Glycyrrhiza* plants probably have acquired some genetic material from other rhizobia in co-evolution with *Glycyrrhiza* and other legume species.

Key words: MLSA, Housekeeping genes, Accessory genes, *Mesorhizobium*, *Glycyrrhiza*.

Introduction

The plant genus *Glycyrrhiza* belongs to the family Fabaceae/Leguminosae and consists of approximately 20 species, among which six species produce a sweet saponin glycyrrhizic acid (glycyrrhizin). Roots and rhizomes of *Glycyrrhiza glabra*, *G. uralensis* and *G. inflata* are considered as licorice, and are used extensively in food, tobacco, cosmetics, and medicine industries [17, 20]. *Glycyrrhiza* species can be found on all continents but Antarctica; and are mostly endemic to Eurasia. *G. glabra* is a Mediterranean species which grows also in Iran, Iraq, Central Asia and the north-western part of China, whereas the species *G. uralensis* is found in China, Central Asia, and Mongolia. *Glycyrrhiza* spp. grow on dry grassy plains and mountainsides in the north-western provinces of China. Even though medicinal properties of *Glycyrrhiza* spp. were studied extensively, over 1500 reports exist on the compound glycyrrhizin, the biology and ecology of the plant species are not well known [15, 14, 20, 33].

Glycyrrhiza spp. can establish symbiotic associations with a wide range of root-nodule bacteria, which in symbiosis reduce atmospheric dinitrogen gas (N₂) to ammonia. At least three symbiotic rhizobial genera, *Mesorhizobium*, *Ensifer* (syn. *Sinorhizobium*) and *Rhizobium* were found to be associated with *Glycyrrhiza* spp. [20]. The genera *Rhizobium* and *Ensifer* are classified in the family *Rhizobiaceae* whereas the genus *Mesorhizobium* consisting of 30 species is the member of the family *Phyllobacteriaceae* (<http://www.bacterio.net>).

Li et al. [20] studied 159 endophytic bacterial isolates from nodules of wild *Glycyrrhiza* growing on 40 sites in central and north-western China. The results of amplified fragment length polymorphism (AFLP) genomic fingerprinting, and partial sequences of the 16S rRNA gene as well as nodulation tests indicated that out of 159 endophytic strains 115 represented *Alphaproteobacteria*, involving the genera *Mesorhizobium* (57 strains), *Rhizobium* (25), *Ensifer* (*Sinorhizobium*) (11), *Phyllobacterium* (6) and *Agrobacterium* (16). Thus, at least, representatives of all these genera can colonize

Glycyrrhiza nodules in China. Based on symbiotic properties, five *Mesorhizobium* groups (with 57 strains) represented the true symbionts of *G. glabra* and *G. uralensis*. Since the 16S rRNA gene is too conserved to allow close species separation [22, 23, 24], and only partial sequences of 16S rRNA gene (*rrs*) of representative strains of each AFLP subgroups were studied by Li et al. [20], further phylogenetic studies were required to resolve the taxonomy of these isolates. Multilocus sequence analysis (MLSA) method is considered as a straightforward method to study the phylogeny and taxonomy of rhizobia [22, 23, 24, 25].

Aiming at determining the accurate taxonomic position of 29 mesorhizobial strains collected from nodules of *Glycyrrhiza* spp. growing in central and north-western China, we performed multilocus sequence analysis (MLSA) for 59 mesorhizobial strains, including 29 *Glycyrrhiza* test strains and 30 type strains of the *Mesorhizobium* species, since multilocus sequence analysis (MLSA) method is considered as a straightforward method to study the phylogeny and taxonomy of rhizobia [22, 23, 24, 25]. Three housekeeping genes, namely 16S rRNA (*rrs*), *recA* and *rpoB*, coding for 16S ribosomal RNA, recombinase A, and RNA polymerase beta subunit, respectively were used in this study. Furthermore, we also investigated the phylogeny of three symbiotic genes of all the 59 mesorhizobial strains, involving nodulation genes *nodA* and *nodC* and the nitrogenase encoding gene *nifH*.

Material and Methods

Bacterial strains and DNA isolations

59 mesorhizobial strains studied here (Table S1) included 29 mesorhizobial strains isolated from *Glycyrrhiza glabra* (10 strains), *G. uralensis* (14) and unidentified *Glycyrrhiza* species (5), and 30 type strains of the *Mesorhizobium* species, in which *Mesorhizobium camelthorni* HAMBI 3020^T, *M. muleiense* HAMBI 3264^T, *M. qingshengii* HAMBI 3277^T, *M. robiniae* HAMBI 3082^T, *M. sangaii* HAMBI 3318^T and *M. shangrilense* HAMBI 3050^T were obtained from the HAMBI Culture Collection, University of Helsinki. The bacterial samples were grown on yeast mannitol agar medium at 28°C for two-three days [27]. For DNA isolation, single colonies of the bacteria were cultured in 5 ml of tryptone-yeast extract broth. Genomic DNA was isolated from each strain using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.) and was preserved at -20°C.

PCR amplification and gene sequencing

Three housekeeping genes (*rrs-recA-rpoB*) and three symbiotic genes (*nodA*, *nodC* and *nifH*) of the *Glycyrrhiza* test strains and the *rpoB* gene of six reference strains (not available in database) were sequenced in the current work. PCR amplification and sequencing were performed by using the

103 primers listed in Table S2. The sequences of the mentioned genes for most of the type strains of
104 *Mesorhizobium* species were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank>). The
105 accession numbers of the studied housekeeping and symbiotic gene sequences are listed in Table S1.

106 **Phylogenetic analyses of the sequences**

107 The sequences were aligned by MUSCLE software at EML-EBI [5] and were edited with program
108 BioEdit version 7.0.5.3 [10]. The best-fit nucleotide substitution model of each gene was selected
109 applying MEGA6 [29]. The concatenated gene tree (*rrs-atpD-rpoB*) of altogether 59 mesorhizobial
110 strains was constructed using maximum likelihood (ML) and Bayesian interference. The ML tree of
111 the combined genes (*rrs-atpD-rpoB*) was constructed with 1,000 bootstrap replicates in MEGA6.
112 The mean distance between the groups of the test and reference strains and the distances between the
113 species were computed by MEGA6. The algorithm Metropolis-coupled Markov chain Monte Carlo
114 (MCMCMC) for 2×10^6 generations was run twice with MrBayes 3.2 for the combined sequences
115 dataset [26]. The generated Bayesian traces and trees were visualized by Tracer v1.5
116 (<http://tree.bio.ed.ac.uk/software/tracer/>) and FigTree v1.4.0
117 (<http://tree.bio.ed.ac.uk/software/figtree/>). The ML individual gene trees of the 16S rRNA, *nodA*,
118 *nodC*, and *nifH* loci were constructed with 1,000 bootstrap replicates in MEGA6.

119 **Results**

120 **Analyses of the housekeeping genes**

121 The phylogeny of the *rrs* gene sequence (1247 bp) of 59 mesorhizobial strains demonstrated that the
122 29 mesorhizobial test strains were placed in five different clusters (Fig. S1). The ML tree of the
123 combined three housekeeping genes (*rrs-recA-rpoB*) (Fig. 1) positioned the 29 test strains in seven
124 separate clades. In addition, the Bayesian analysis returned similar topologies (not shown).
125 Eight strains isolated from Xinjiang region (Chahan, Hejing, and Ziniqan towns) formed a distinct
126 clade (clade C) with a high bootstrap support (BS=100%) that accommodates none of the reference
127 strains. Seventeen Chinese test strains clustered with *M. tianshanense*, *M. temperatum*, *M. muleiense*,
128 and *M. alhagi* with high confidence (BS>85%), representing the groups A, D, E, and G respectively.
129 The strain NWGS09 was placed close to *M. tianshanense* and *M. gobiense*. Based on the sequences
130 of two protein-coding housekeeping genes (*recA-rpoB*), the pairwise ANI between eight
131 *Mesorhizobium* species were over 96%. The strain NWSX24 was placed close to the clade that
132 accommodates *M. temperatum* and three test strains (clade D). The strain NWNX05, isolated from
133 *Glycyrrhiza* sp. in Ningxia, was grouped with *M. gobiense* supported by 100% of bootstrap value.
134 Strain NWGS05, isolated from *Glycyrrhiza* sp. in Lingtai, Gansu, was clustered with *M. amorphae*
135 (BS=100%). Based on the sequences of two protein-coding housekeeping genes (*recA-rpoB*), the
136 pairwise ANI between the following four pairs of species, *M. qingshengii* and *M. huakuui*, *M.*

137 *silamurunense* and *M. shonense*, *M. tianshanense* and *M. gobiense*, and *M. alhagi* and *M. camelthorni*
138 were over 96%.

139
140
141

142 **Analyses of the individual accessory genes**

143 Based on the analyses of the *nodA* gene sequences (Fig. S2 and Table 1), 28 test strains were placed
144 in four main groups (A, B, C and E). Interestingly, the strains of clade B were placed separately from
145 the reference strains. The *nodA* sequences of the species *M. tianshanense* and *M. temperatum* were
146 grouped with 15 test strains, though neither of the clusters was strongly bootstrapping supported
147 (BS<75%). In the *nodA* gene tree, the species *M. alhagi* formed a well-supported clade (E) with five
148 Chinese test strains alongside the species *M. camelthorni*.

149 In the *nodC* gene tree, 28 Chinese test strains were positioned in seven clusters (A-G). The clades A,
150 B, E, and G accommodated the type strains of the species *M. tianshanense*, *M. gobiense*, *M.*
151 *temperatum*, *M. camelthorni* and *M. alhagi*. In the *nifH* gene tree, the Chinese test strains were placed
152 in nine lineages. Clade A was positioned close to *M. tianshanense* and strain NWGS09. In the three
153 accessory gene trees, the Chinese test strains did not cluster with the same reference species, with
154 which they were grouped in the concatenated housekeeping gene tree. Among the type strains, the
155 species *M. alhagi* was positioned close to *M. camelthorni* in the three studied accessory gene trees.

156
157
158

159 **Discussion**

160 The results of the present MLSA of three housekeeping genes (*rrs-recA-rpoB*) demonstrated that 20
161 test strains isolated mostly from the plant species *G. glabra* and *G. uralensis* were placed in the clades
162 A, B, D, E, F, and G, which represent the species *M. tianshanense*, *M. gobiense*, *M. temperatum*, *M.*
163 *muleiense*, *M. amorphae*, *M. alhagi*, and *M. camelthorni*, respectively. All above mentioned seven
164 reference species were originally isolated in China, from a wide range of legumes. Eight test strains
165 representing clade C, namely NWXJ21, NWXJ27, NWXJ31, NWXJ 33, NWXJ36, NWJ39,
166 NWXJ40, and NWXJ43, did not cluster with any of the 30 described *Mesorhizobium* species,
167 implying that they could belong to a putative novel species of the genus *Mesorhizobium*.

168 Konstantinidis et al. [16] proposed an ANI of 96% corresponds to a cut-off of 70% DNA–DNA
169 hybridization for species delineation, whilst Martens et al. [22] proposed an ANI of 97.3% for species
170 delineation within the genus *Ensifer*. Moreover, the study by Figueras et al. [6] recommended a cut-

171 off score of 95% for species delineation. These variations demonstrated that the cut-off ANI values
172 may change when different genes were used. The concatenated gene tree and the ANI analyses in this
173 study showed that the following four pairs of species, *M. qingshengii*-*M. huakuui*, *M. silamurunense*-
174 *M. shonense*, *M. tianshanense*-*M. gobiense*, and *M. alhagi*-*M. camelthorni* might need to be merged
175 into four species. However, it is worth noting that in this study, we only analysed three housekeeping
176 genes, which might not be a sufficient number of genes to delineating new species and species
177 combinations. Merging these eight species requires a more comprehensive phylogenetic study of
178 mesorhizobia preferably by comparing whole-genome sequences. In the concatenated housekeeping
179 gene tree, strain NWGS09, which was isolated from *Glycyrrhiza* sp. in Gansu, was positioned
180 between the two species *M. tianshanense* and *M. gobiense*. However, since the two species *M.*
181 *tianshanense* and *M. gobiense* are very similar to each other, strain NWGS09 cannot represent a new
182 species. The results of MLSA confirmed the groups formed based on AFLP, a whole-genome
183 fingerprinting method, by Li et al. [20], with the exception of the grouping of strains NWSX22,
184 NWGS09, and NWNX05.

185 In the *nodA* gene tree, 15 test strains (clades A and C) were clustered with two species, *M.*
186 *tianshanense* and *M. temperatum*. The former species was isolated from *G. uralensis* in the Xinjiang
187 of China and the latter one was isolated from *Astragalus adsurgens* in northern regions of China [3,
188 7]. However, Based on the *nodC* sequences, the 15 test strains (clades A, B, E, and G) clustered with
189 the species *M. tianshanense*, *M. gobiense*, *M. temperatum*, *M. camelthorni*, and *M. alhagi*. The
190 species *M. tianshanense*, *M. gobiense*, *M. temperatum*, *M. alhagi*, and *M. camelthorni* are native
191 species of China. The species *M. alhagi*, and *M. camelthorni* were isolated from the plant genus
192 *Alhagi* in Xinjiang, and *M. gobiense* was isolated from the genus *Oxytropus*. However, the two
193 species *M. alhagi*, and *M. camelthorni* were also able to nodulate *Glycyrrhiza inflata* and *M. gobiense*
194 could form nodules on *G. uralensis* respectively [1, 2, 11]. In the *nodA* and *nodC* gene trees, several
195 test strains were grouped close to the species *M. tianshanense*, which was also isolated from the plant
196 genus *Glycyrrhiza* in China [3].

197 The phylogeny of symbiotic genes is often incongruent with the housekeeping genes [28, 31]. This
198 was also observed in our study for most of the strains isolated from *Glycyrrhiza* species. Albeit the
199 representatives of clades A, C and G of the combined housekeeping gene tree grouped together also
200 in the *nodA*, *nodC* and *nifH* gene trees, the positions of the clades in the three symbiotic gene trees
201 and the concatenated housekeeping gene tree were not similar, indicating that in the case of
202 mesorhizobia, the phylogeny of the tested accessory genes were not congruent with housekeeping
203 genes in our study.

204 Three strains of clade E (NWGS01, NWGS03, NWSX06) of the MLSA tree were grouped together
 205 in the *nodA*, *nodC* and *nifH* gene trees. In the concatenated housekeeping gene tree, these three strains
 206 formed a monophyletic group (clade E) with two other test strains (NWSX22 and NWSX23) and *M.*
 207 *muleiense*. However, these five test strains were not clustered together in the accessory gene trees.
 208 The species *M. muleiense* was placed within the clade E (MLSA), and close to the species *M.*
 209 *temperatum* in the concatenated gene tree, whereas, in the *nodC* and *nifH* gene trees, *M. muleiense*
 210 was placed distinctly from the other strains of clade E (MLSA) and *M. temperatum* (the sequence of
 211 *nodA* gene is missing for *M. muleiense*). Strain NWSX19 (representing MLSA clade D) appeared
 212 more similar to NWSX22 (representing MLSA clade E) in the *nodA* and *nodC* gene trees, but not in
 213 the *nifH* gene tree.

214 To find out whether the variation in genomic fingerprint AFLP patterns exhibits specific
 215 biogeographical patterns, Li et al. [20] performed multivariate analyses of AFLP patterns of 159
 216 bacterial strains isolated from nodules of *Glycyrrhiza* spp. in China. It showed that plant species and
 217 geographic location explained only a small part of the total variation in AFLP patterns (14.4%).
 218 However, strains isolated from *G. glabra* were clearly separated from those obtained from *G.*
 219 *uralensis*, and the strains obtained from Central China were well separated from those originating
 220 Xinjiang, indicating host preference and regional endemism [20].

221 In the current work, the clades of the combined housekeeping gene tree accommodated strains
 222 isolated from the same regions, with the exception of clade E. The clade E contains strains from two
 223 different regions, Gansu and Shaanxi, which are neighbor provinces. In the case of the strains of clade
 224 E which were isolated from the plant *G. uralensis* in two neighbor provinces (Shaanxi and Gansu),
 225 three strains (NWGS01, NWGS03, and NWSX06) were placed distinctly from (NWSX22 and
 226 NWSX23) in the accessory gene trees. All strains isolated from the same provinces did not form a
 227 monophyletic lineage either in the combined housekeeping gene tree or in the symbiotic gene trees.
 228 Wernegreen and Riley [31] also reported that geographical positions of rhizobial strains have a
 229 relatively poor fit across their phylogenies at the species level in comparison with host plant genus.

230 *Glycyrrhiza glabra* grows mainly in Xinjiang (the northwest), whereas *G. uralensis* grows in all parts
 231 of northern China [15]. In the study of Li et al. [20], the geographic distribution of these two
 232 *Glycyrrhiza* species sampled was similar. Based on genetic markers, Kondo et al. [15] concluded that
 233 *G. glabra* is more closely related to *G. inflata* than to *G. uralensis*, *G. glabra* and *G. inflata* being
 234 equidistant to *G. uralensis*. However, the genetic difference between them is not large because *G.*
 235 *uralensis* can hybridize with *G. glabra* and *G. inflata* [13, 15]. This close genetic relationship is
 236 supposed to explain why most nodulating mesorhizobial strains were capable of forming nodules on
 237 both *G. uralensis* and *G. glabra* [20]. Unlike the report on mesorhizobia isolated from *Acacia* and

238 *Sesbania sesban* tree species [4], where mesorhizobia isolated from the same plant were grouped
239 together in MLSA based on housekeeping genes (*gltA*, *gyrB*, *recA*, and *rpoB*), clustering of the strains
240 isolated from *Glycyrrhiza* was not observed in this work. The differences between these two results
241 might be due to the plant genera *Acacia* (Mimosoideae) and *Sesbania* (Papilioinoideae) being
242 distantly related, whilst *G. glabra* and *G. uralensis* have a very close relationship and share similar
243 growth conditions.

244
245 At a higher phylogenetic level, in the symbiotic gene trees, most of the mesorhizobia isolated from
246 China were placed separately from the *Mesorhizobium* species isolated from other countries than
247 China. The plant species *G. glabra* is a Mediterranean species, and the species *G. uralensis* is
248 probably originated from the northern or north-western neighbours of China. Therefore, the Chinese
249 mesorhizobia involved in symbiosis with the plant of *G. uralensis* and *G. uralensis* might have
250 acquired some genetic material from other rhizobia in co-evolution with *Glycyrrhiza* and other
251 legume species. Probably, the same *Mesorhizobium* species can induce nitrogen-fixing nodules on
252 many host plants. A similar hypothesis was proposed by Tan et al. [30] about the co-evolution of some
253 rhizobia and Carmichaelinae plants in New Zealand. Likewise, Wernegreen and Riley [31] pointed
254 out that mapping of host plant genus across the *Mesorhizobium nod* phylogeny is less clear in
255 comparison with the genera *Rhizobium* and *Ensifer*. Based on the phylogenetic analyses of the three
256 symbiotic genes, the Chinese test strains were grouped with bacteria that were isolated from the plant
257 genera *Alhagi*, *Astragalus*, *Amorpha*, *Oxytropis*, *Glycyrrhiza* and *Caragana* in China, and from the
258 genus *Anthyllis* in France. These genera belong to the inverted repeat-lacking clade (IRLC) of
259 legumes; with the exception of the genera *Amorpha* and *Anthyllis*, which are positioned in the plant
260 clades Dalbergioids s.l. and Robinioids [21, 32]. However, the close relationships of the plants could
261 affect that several *Mesorhizobium* species can form at least non-fixing nodules on these plants.

262 To conclude, the current work showed that eight *Mesorhizobium* strains isolated from *G. uralensis*
263 and *G. glabra* in central and north-western China belong to a putative new species. Whole-genome
264 sequencing of more mesorhizobial species will contribute to ascertaining the taxonomic positions of
265 the members of the genus *Mesorhizobium*. A genealogy of rhizobia from the gene center of
266 *Glycyrrhiza* could reveal more about the co-evolution of mesorhizobia and *Glycyrrhiza*.

268 **Acknowledgments**

269 We would like to thank Riitta Saastamoinen for providing us the bacterial strains used in this study.
270 This research was funded by the Academy of Finland, Societas Scientiarum Fennica, the Department
271 of Environmental Sciences of the University of Helsinki, and the National Science Foundation of
272 China, and the 973 project of China (2010CB126502).

274 **References**

- 275 [1] Chen, W., Zhu, W., Bontemps, C., Young, J.P.W., Wei, G.H. (2010). *Mesorhizobium alhagi*
 276 sp. nov., isolated from wild *Alhagi sparsifolia* in north-western China. Int. J. Syst. Evol. Microbiol.
 277 60,958-962 .
- 278 [2] Chen, W., Zhu, W., Bontemps, C., Young, J.P.W., Wei, G. (2011). *Mesorhizobium*
 279 *camelthorni* sp. nov., isolated from *Alhagi sparsifolia*. Int. J. Syst. Evol. Microbiol. 61,574-579
- 280 [3] Chen, W., Wang, E., Wang, S., Li, Y., Chen, X., Li, Y. (1995). Characteristics of *Rhizobium*
 281 *tianshanense* sp. nov., a Moderately and Slowly Growing Root Nodule Bacterium Isolated from an
 282 Arid Saline Environment in Xinjiang, People's Republic of China. Int. J. Syst. Evol. Microbiol.
 283 45,153-159.
- 284 [4] Degefu, T., Wolde-meskel, E., Frostegård, Å. (2011). Multilocus sequence analyses reveal
 285 several unnamed Mesorhizobium genospecies nodulating *Acacia* species and *Sesbania sesban* trees
 286 in Southern regions of Ethiopia. Syst. Appl. Microbiol. 34,216-226
- 287 [5] Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high
 288 throughput. Nucleic Acids Res. 32, 1792-1797.
- 289 [6] Figueras, M.J., Beaz-Hidalgo, R., Hossain, M.J., Liles, M.R. (2014). Taxonomic Affiliation
 290 of New Genomes Should Be Verified Using Average Nucleotide Identity and Multilocus
 291 Phylogenetic Analysis. Genome Announc. 2,e00927-14.
- 292 [7] Gao, J., Turner, S.L., Kan, F.L., Wang, E.T., Tan, Z.Y., Qiu, Y.H., Gu, J., Terefework, Z.,
 293 Young, J.P.W., Lindström, K., Chen, W.X. (2004). *Mesorhizobium septentrionale* sp. nov. and
 294 *Mesorhizobium temperatum* sp. nov., isolated from *Astragalus adsurgens* growing in the northern
 295 regions of China. Int. J. Syst. Evol. Microbiol. 54,2003-2012.
- 296 [8] Gaunt, M.W., Turner, S.L., Rigottier-Gois, L., Lloyd-Macgilp, S.A., Young, J.P. (2001)
 297 Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. Int.
 298 J. Syst. Evol. Microbiol. 51, 2037-2048.
- 299 [9] Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J., Lopez, R. (2010)
 300 A new bioinformatics analysis tools framework at EMBL–EBI. Nucleic Acids Res. 38, W695-W699.
- 301 [10] Hall T.A., (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis
 302 program for Windows 95/98/NT. Nucl. Acid. Symp. Ser. 41, 95–98.
- 303 [11] Han, T.X., Han, L.L., Wu, L.J., Chen, W.F., Sui, X.H., Gu, J.G., Wang, E.T., Chen, W.X.
 304 (2008). *Mesorhizobium gobiense* sp. nov. and *Mesorhizobium tarimense* sp. nov., isolated from wild
 305 legumes growing in desert soils of Xinjiang, China. Int. J. Syst. Evol. Microbiol. 58,2610-2618.
- 306 [12] Haukka, K., Lindström, K., Young, J.P. (1997). Three Phylogenetic Groups of *nodA* and *nifH*
 307 Genes in *Sinorhizobium* and *Mesorhizobium* Isolates from Leguminous Trees Growing in Africa and
 308 Latin America. Appl. Environ. Microbiol. 64,419-426.
- 309 [13] Hayashi, H., Hattori, S., Inoue, K., Sarsenbaev, K., Ito, M., Honda, G. (2003). Field survey of
 310 *Glycyrrhiza* plants in central Asia (1). Characterization of *G. uralensis*, *G. glabra* and the putative
 311 intermediate collected in Kazakhstan. Biol. Pharm. Bull. 26,867-871.
- 312 [14] Hayashi, H. Sudo, H. (2009). Economic importance of licorice. Plant. Biotechnol. 26,101-104
- 313 [15] Kondo, K., Shiba, M., Yamaji, H., Morota, T., Zhengmin, C., Huixia, P., Shoyama, Y. (2007).
 314 Species identification of licorice using nrDNA and cpDNA genetic markers. Biol. Pharm. Bull.
 315 30,1497-1502
- 316 [16] Konstantinidis, K.T., Ramette, A., Tiedje, J.M. (2006). Toward a More Robust Assessment
 317 of Intraspecies Diversity, Using Fewer Genetic Markers. Appl. Environ. Microbiol. 72,7286-7293.
- 318 [17] Kushiev, H., Noble, A.D., Abdullaev, I., Toshbekov, U. (2005). Remediation of abandoned
 319 saline soils using *Glycyrrhiza glabra*: a study from the Hungry Steppes of Central Asia. Iint. J. Agr.
 320 Sustain. 3,102-113.

- 321 [18] Laguerre, G., Nour, S.M., Macheret, V., Sanjuan, J., Drouin, P., Amarger, N. (2001).
 322 Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic
 323 relationship among *Phaseolus vulgaris* symbionts. Microbiol. 147,981-993.
- 324 [19] Laranjo, M., Alexandre, A., Oliveira, S. (2014). Legume growth-promoting rhizobia: an
 325 overview on the *Mesorhizobium* genus. Microbiol. Res. 169,2-17.
- 326 [20] Li, L., Sinkko, H., Montonen, L., Wei, G., Lindström, K., Räsänen, L.A. (2012).
 327 Biogeography of symbiotic and other endophytic bacteria isolated from medicinal *Glycyrrhiza*
 328 species in China. FEMS Microbiol. Ecol. 79,46-68.
- 329 [21] Lock M. & Schrire, B.D. 2005. Tribe Galegeae. In: Lewis, G.P. et al. (Eds.), Legumes of the
 330 World, Royal Botanic Gardens, Kew, pp. 475-487.
- 331 [22] Martens, M., Dawyndt, P., Coopman, R., Gillis, M., De Vos, P., Willems, A. (2008).
 332 Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10
 333 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). Int. J. Syst. Evol.
 334 Microbiol. 58,200-214 .
- 335 [23] Martens, M., Delaere, M., Coopman, R., De Vos, P., Gillis, M., Willems, A. (2007).
 336 Multilocus sequence analysis of *Ensifer* and related taxa. Int. J. Syst. Evol. Microbiol. 57,489-503.
- 337 [24] Mousavi, S.A., Österman, J., Wahlberg, N., Nesme, X., Lavire, C., Vial, L., Paulin, L., De
 338 Lajudie, P., Lindström, K. (2014). Phylogeny of the *Rhizobium*–*Allorhizobium*–*Agrobacterium* clade
 339 supports the delineation of *Neorhizobium* gen. nov. Syst. Appl. Microbiol. 37,208-215.
- 340 [25] Mousavi, S.A., Willems, A., Nesme, X., de Lajudie, P., Lindström, K. (2015). Revised
 341 phylogeny of *Rhizobiaceae*: Proposal of the delineation of *Pararhizobium* gen. nov., and 13 new
 342 species combinations. Syst. Appl. Microbiol. 38,84-90.
- 343 [26] Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B.,
 344 Liu, L., Suchard, M.A., Huelsenbeck, J.P. (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic
 345 Inference and Model Choice across a Large Model Space. Syst. Biol. 61, 539-42.
- 346 [27] Somasegaran, P., and Hoben, H.J. (2012). Handbook for rhizobia: methods in legume-
 347 *Rhizobium* technology Springer Science & Business Media).
- 348 [28] Suominen, L., Roos, C., Lortet, G., Paulin, L., Lindström, K. (2001). Identification and
 349 structure of the *Rhizobium galegae* common nodulation genes: evidence for horizontal gene transfer.
 350 Mol. Biol. Evol. 18,907-916 .
- 351 [29] Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013). MEGA6: Molecular
 352 Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30,2725-2729.
- 353 [30] Tan, H.W., Weir, B.S., Carter, N., Heenan, P.B., Ridgway, H.J., James, E.K., Sprent, J.I.,
 354 Young, J.P.W., Andrews, M. (2012). Rhizobia with 16S rRNA and *nifH* similar to *Mesorhizobium*
 355 *huakuii* but Novel *recA*, *glnII*, *nodA* and *nodC* genes are symbionts of New Zealand *Carmichaelinae*.
 356 [31] Wernegreen, J.J. Riley, M.A. (1999). Comparison of the evolutionary dynamics of symbiotic
 357 and housekeeping loci: a case for the genetic coherence of rhizobial lineages. Mol. Biol. Evol. 16,98-
 358 113.
- 359 [32] Wojciechowski, M.F., Lavin, M., Sanderson, M.J. (2004). A phylogeny of legumes
 360 (Leguminosae) based on analysis of the plastid *matK* gene resolves many well-supported subclades
 361 within the family. Am. J. Bot. 91,1846-1862.
- 362 [33] Zimnitskaya, S. (2009). State of the reproductive system of populations of species of the genus
 363 *Glycyrrhiza* L.(Fabaceae). Contemp. Probl. Ecol. 2,392-395.

371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398

Table 1. List of the mesorhizobial test strains studied, plant and place of isolation, and the position of the test strains in the combined gene tree, AFLP, and the individual accessory gene trees.

Strain	Host	Isolation site*	MLSA	AFLP**	<i>nodA</i>	<i>nodC</i>	<i>nifH</i>
NWXJ16	<i>G. uralensis</i>	Xinjiang, Bachu	G	4a	E	G	***
NWXJ42	<i>G. uralensis</i>	Xinjiang, Hejing	G	4a	E	G	I
NWXJ02	<i>G. uralensis</i>	Xinjiang, Bachu	G	4a	E	G	I
NWXJ08	<i>G. uralensis</i>	Xinjiang, Bachu	G	4a	E	G	I
NWXJ18	<i>G. uralensis</i>	Xinjiang, Bachu	G	4a	E	G	I
NWXJ33	<i>G. glabra</i>	Xinjiang, Chahan	C	5e	B	C	A
NWXJ39	<i>G. glabra</i>	Xinjiang, Chahan	C	5e	B	C	A
NWXJ43	<i>G. uralensis</i>	Xinjiang, Hejing	C	5e	B	C	A
NWXJ36	<i>G. glabra</i>	Xinjiang, Chahan	C	5e	B	C	A
NWXJ27	<i>G. glabra</i>	Xinjiang, Ziniquan	C	5e	B	C	A
NWXJ31	<i>G. glabra</i>	Xinjiang, Ziniquan	C	5e	B	C	A
NWXJ21	<i>G. glabra</i>	Xinjiang, Ziniquan	C	5e	B	C	A
NWXJ40	<i>G. glabra</i>	Xinjiang, Chahan	C	5e	B	C	A
NWGS01	<i>G. uralensis</i>	Gansu, Huanxian	E	3c	A	F	B
NWGS03	<i>G. uralensis</i>	Gansu, Huanxian	E	3c	A	F	B
NWSX06	<i>G. uralensis</i>	Shaanxi, Ziwuling	E	3c	A	F	B
NWSX22	<i>G. uralensis</i>	Shaanxi, Yangling	E	5b	C	D	D
NWSX23	<i>G. uralensis</i>	Shaanxi, Yangling	E	3c	C	B	D
NWSX19	<i>Glycyrrhiza</i> sp.	Shaanxi, Yanan	D	3a	C	D	F
NWSX20	<i>Glycyrrhiza</i> sp.	Shaanxi, Yanan	D	3a	C	E	G
NWSX24	<i>G. uralensis</i>	Shaanxi, Yangling	D	3a	C	A	E
NWSX25	<i>G. uralensis</i>	Shaanxi, Yangling	D	3a	C	B	E
NWXJ28	<i>G. glabra</i>	Xinjiang, Ziniquan	A	3b	A	A	E
NWXJ32	<i>G. glabra</i>	Xinjiang, Ziniquan	A	3b	A	A	E
NWXJ25	<i>G. glabra</i>	Xinjiang, Ziniquan	A	3b	A	A	E
NWXJ30	<i>G. glabra</i>	Xinjiang, Ziniquan	A	3b	A	A	E

NWGS09	<i>Glycyrrhiza</i> sp.	Gansu, Chongxing	A-B	3c	A	B	A-B
NWNX05	<i>Glycyrrhiza</i> sp.	Ningxia, Zhongwei	B	3b	A	A	C
NWGS05	<i>Glycyrrhiza</i> sp.	Gansu, Lingtai	F	5a	D	***	H

* Province, site. **AFLP subgroups are reported from the study by Li et al. [20]. ***missing data.

Figure 1. Phylogenetic tree of three concatenated housekeeping genes (*rrs-recA-rpoB*) of 59 mesorhizobial strains, constructed based on a maximum likelihood analysis. The genus name *Mesorhizobium* is abbreviated as *M.* The type strains are shown by a “T” at the end of each strain code.