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1 **Title:** Average nucleotide identity of genome sequences supports the description of  
2 *Rhizobium lentis* sp. nov., *Rhizobium bangladeshense* sp. nov. and *Rhizobium binae*  
3 sp. nov. from lentil (*Lens culinaris*) nodules  
4

5 **Running title:** Rhizobia nodulating lentil

6 **Content category:** New Taxa Proteobacteria

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26  
27

28 **Strain reference and accession number for type strain:**  
29

30 ***Rhizobium lentis*:** Type strain is BLR27<sup>T</sup> (=LMG28441<sup>T</sup>=DSMZ29286<sup>T</sup>). Accession numbers for 16S  
31 rRNA: JN648905; *atpD*: JN648941; *recA*: JN649031; *glnII*: JN648976 and whole genome sequences:  
32 PRJEB7125 (<http://www.ebi.ac.uk/ena/data/view/PRJEB7125>).  
33

34 ***Rhizobium bangladeshense*:** The type strain is BLR175<sup>T</sup> (= LMG 28442<sup>T</sup> = DSMZ 29287<sup>T</sup>).  
35 Accession numbers for 16S rRNA: JN648931; *atpD*: JN648967; *recA*: JN649057; *glnII*: JN648979 and  
36 whole genome sequences:PRJEB7125 (<http://www.ebi.ac.uk/ena/data/view/PRJEB7125>).  
37

38 ***Rhizobium binae*:** The type strain is BLR195<sup>T</sup>(=LMG28443<sup>T</sup>=DSMZ29288<sup>T</sup>). Accession numbers for  
39 16S rRNA: JN648932; *atpD*: JN648968; *recA*: JN649058; *glnII*: JN648980 and and whole genome  
40 sequences:PRJEB7125 (<http://www.ebi.ac.uk/ena/data/view/PRJEB7125>).  
41

42 **Abstract**

43 Rhizobial strains isolated from effective root nodules of field-grown lentil (*Lens culinaris*)  
44 from different parts of Bangladesh were previously analyzed using four housekeeping  
45 genes (16S rRNA, *recA*, *atpD* and *glnII*) and three nodulation genes (*nodA*, *nodC* and  
46 *nodD*), DNA fingerprinting and phenotypic characterization. Analysis of housekeeping  
47 genes and DNA fingerprint indicated that the isolates belonged to three new clades in  
48 the genus *Rhizobium*. In the present study, a representative strain from each clade was  
49 further characterized by cellular fatty acid compositions, carbon substrate utilization  
50 pattern, DNA-DNA hybridization and average nucleotide identity (ANI) analyses from  
51 whole genome sequences. The DNA-DNA hybridization showed 50 – 56 % relatedness  
52 to their closest relatives (*Rhizobium etli* and *Rhizobium phaseoli*) and 50 – 60 %  
53 relatedness to each other. These results were further supported by average nucleotide  
54 identity values, based on genome sequencing, which were 87 – 92 % with their close  
55 relatives and 87 – 88 % with each other. On the basis of these results, three novel  
56 species, *Rhizobium lentis* sp. nov. (type strain BLR27<sup>T</sup>), *Rhizobium bangladeshense* sp.  
57 nov. (type strain BLR175<sup>T</sup>) and *Rhizobium binae* sp. nov. (type strain BLR195<sup>T</sup>) are  
58 proposed. These species share common nodulation genes (*nodA*, *nodC* and *nodD*) that  
59 are similar to those of the symbiovar *viciae*.

60

61

62 Key words: Rhizobia, lentil, DNA-DNA relatedness, genome sequencing, average  
63 nucleotide identity

64

65

66

67 Rhizobia are nodule-forming nitrogen fixing bacteria that belong to the bacterial phylum  
68 Proteobacteria. Rhizobia can satisfy the nitrogen requirement of legumes by effective  
69 symbiosis with these plants and therefore are important bacteria for supporting plant  
70 growth and for environmental protection. About 180 species of nodule-forming bacteria  
71 in 12 different genera have been described. *Rhizobium* is an important genus of  
72 rhizobia and more than 80 species are currently included in this genus  
73 (<http://www.bacterio.net/rhizobium.html>). Among agricultural legume crops, lentil (*Lens*  
74 *culinaris*) is one of the oldest and remains very popular all over the world; it forms an  
75 effective symbiosis with rhizobia. *Rhizobium leguminosarum* symbiovar *viciae* (Rlv) is  
76 the main symbiont of the legume tribe Viciae (Hou *et al.*, 2009; Laguerre *et al.*, 2003;  
77 Mutch & Young, 2004; Santillana *et al.*, 2008; Tian *et al.*, 2010 and many others), to  
78 which lentils belong, although other species, including *Rhizobium pisi*, *Rhizobium fabae*  
79 and *Rhizobium laguerreae* (Ramirez-Bahena *et al.*, 2008; Saïdi *et al.*, 2014; Tian *et al.*,  
80 2008), have also been described. Previous studies on rhizobia from lentil root nodules  
81 from different geographical locations revealed that Rlv is the major symbiont of lentil  
82 (Geniaux & Amarger, 1993; Hynes & O'Connell, 1990; Laguerre *et al.*, 1992; Materon *et*  
83 *al.*, 1995; Mowad & Beck, 1991). However, in our previous studies (Rashid *et al.*, 2012  
84 & 2014) we identified three new rhizobial clades / lineages in Bangladesh by multi locus  
85 sequence analysis, phenotypic and DNA fingerprint analyses. DNA-DNA hybridization  
86 (DDH) is an important experimental method to detect similarity between two genome  
87 sequences and a DDH value of 70% is the threshold value for bacterial species  
88 demarcation (McCarthy & Boltan, 1963). Wayne *et al.*, (1987) agreed that “the complete  
89 deoxyribonucleic acid (DNA) sequence would be the reference standard to determine  
90 phylogeny and that phylogeny should determine taxonomy”, which was an impracticable  
91 goal at the time but is increasingly feasible (Chan *et al.*, 2012). The approach known as  
92 Average Nucleotide Identity (ANI, Konstantinidis & Tiedje, 2005) provides relatedness  
93 information for prokaryotes at the whole genome level. ANI values equal to 95 – 96 %,  
94 calculated from pair-wise comparisons of shared sequences between two genomes,  
95 provides an equivalent value to DDH values of 70 %, which is the threshold value for  
96 bacterial species demarcation (Chan *et al.*, 2012; Goris *et al.*, 2007; Konstantinidis &  
97 Tiedje, 2005; Wayne *et al.*, 1987).

99 Rhizobial strains were isolated from field-grown lentil root nodules from different parts of  
100 Bangladesh in 2009. Detailed descriptions of the strains (isolation localities, isolation  
101 procedure; identities; phylogenetic and population analysis; DNA fingerprint analysis  
102 and phenotypic characterization) are available in Rashid *et al.* (2012; 2014). DNA  
103 fingerprint analysis using ERIC-PCR, phylogeny and population analysis of *recA*, *atpD*  
104 and *glnII* genes strongly suggested that three clades were clearly distinct from all  
105 described *Rhizobium* species and belong to three novel species. In present study, we  
106 extended the phylogeny of the previously described 15 strains by including recently  
107 described rhizobial species. Three strains from these three lineages (BLR27<sup>T</sup>, BLR175<sup>T</sup>  
108 and BLR195<sup>T</sup> from lineages I, II and III, respectively) were further characterized in this  
109 study by cellular fatty acid compositions, DNA-DNA hybridization and average  
110 nucleotide identity (ANI) to determine whether the proposal of new species was justified.

111

112 We described three new lineages in the genus *Rhizobium* from effective root nodules of  
113 lentils from Bangladesh in 2012 (Rashid *et al.*, 2012). Since then, several additional  
114 species have been described in this genus. Therefore, in present study the 16S-rRNA,  
115 *recA* and *atpD* gene sequences from 15 strains from our previous study were compared  
116 with recently described species to assess their taxonomic status. The sequences were  
117 obtained from NCBI and aligned with the Clustal X (Thompson *et al.*, 1997) in BioEdit  
118 (Hall, 1999). Phylogenetic trees were reconstructed using the neighbour-joining (NJ,  
119 Saitou & Nei, 1987) and maximum likelihood (ML, Rogers & Swofford, 1998) methods in  
120 MEGA version 5 (Tamura *et al.*, 2011). For sequence evolution, general time reversible  
121 (GTR) model with gamma distribution was used in ML analysis. Bootstrap support for  
122 each node was evaluated with 1000 replicates. Trees were rooted using *Mesorhizobium*  
123 as outgroup. The 16S rRNA gene sequences from the strains from proposed new  
124 species (*R. lentis*, *R. bangladeshense* and *R. binae*) were very similar and clustered  
125 with type strains of *R. etli* CFN42<sup>T</sup>, *R. phaseoli* CIAT652, *R. fabae* CCBAU33202<sup>T</sup>, *R.*  
126 *pisi* DSM30132<sup>T</sup>, *R. leguminosarum* USDA2370<sup>T</sup>, *R. laguerreae* FB206<sup>T</sup> (Fig. 1). The  
127 analysis of *recA*, *atpD* and *gln II* genes, and their concatenated sequences in our  
128 previous study (Rashid *et al.*, 2012) and the concatenated sequences of *recA* and *atpD*

129 genes in Fig. 2 revealed that the proposed species formed three separate clades /  
130 lineages and the closest relatives were *R. etli*, and *R. phaseoli*. Moreover, *Rhizobium*  
131 sp. strain ESC1110, isolated from *Phaseolus vulgaris* from Hispaniola Island (Díaz-  
132 Alcántara *et al.*, 2014) was closely related to *R. lentis* (Fig. 2).

133

134 Phylogenetic analysis of lentil rhizobia from Bangladesh in the present and previous  
135 studies showed that they belonged to the *Rhizobium leguminosarum* species complex,  
136 based on 16S rRNA gene sequences, but this gene did not provide a clear taxonomic  
137 identification since the sequences were >99% identical among these strains and with  
138 representatives of more than one closely related species. The phylogeny of the  
139 housekeeping genes *recA* and *atpD* was first used by Gaunt *et al.* (2001) and *glnII* gene  
140 by Turner & Young (2000); Stepkowski *et al.*, (2005); and Vinuesa *et al.*, (2005) to  
141 delineate the phylogeny of rhizobia and related bacteria with more confidence than was  
142 possible with 16S rRNA alone. These markers have been used widely and successfully  
143 in many studies of rhizobial diversity since then, although additional housekeeping  
144 genes may further improve the reliability of this approach (Martens *et al.*, 2007,  
145 Vinuesa, 2010). Based on a phylogenetic analysis of these three housekeeping protein-  
146 coding genes, the lentil isolates fell into three clades that were distinct enough from  
147 each other and from known species to suggest that they might represent three new  
148 species. The phylogeny of housekeeping genes is particularly useful for placing multiple  
149 new isolates in relation to those described previously, as in this instance. It should be  
150 noted that, although the housekeeping gene phylogenies indicate that the lentil rhizobia  
151 from Bangladesh form three distinct lineages, their nodulation gene sequences do not  
152 reflect these lineages. Instead, the majority of strains, regardless of clade, share  
153 identical sequences for three genes involved in nodulation of the host plant, *nodA*, *nodC*  
154 and *nodD* (Rashid *et al.*, 2012). These, and the variants found in the remaining strains,  
155 fall within the range of variation characteristic of symbiovar *viciae*. The symbiovar is a  
156 key attribute for the description of rhizobia: strains belonging to a symbiovar have  
157 similar nodulation genes and nodulate a similar range of hosts (Rogel *et al.*, 2011).  
158 Importantly, strains that share the same symbiovar need not belong to the same  
159 species, because the nodulation genes are part of the accessory genome (Young *et al.*,

160 2006) and have frequently been subject to horizontal gene transfer within and between  
161 species (Young & Wexler, 1988). In this instance, all the lentil symbionts, regardless of  
162 species, belong to symbiovar *viciae* (Rashid *et al.*, 2012).

163 Genetic diversity of the strains within each of the three clusters was assessed by high  
164 resolution ERIC-PCR, showing that the strains that belong to same cluster were not  
165 clonal (Rashid *et al.*, 2012). For describing new rhizobial species, uncorrected genetic  
166 distances (similarity levels) correspond to an important parameter. Sequences of the  
167 *recA* and *atpD* genes of the three proposed species differed by 3.8 – 11.4% from those  
168 of the type strains of all other species in this clade of *Rhizobium* (Table S2). On the  
169 other hand, differences among strains within each species never exceeded 1.0% (Table  
170 S3), even though ERIC-PCR demonstrated that all the strains were genetically distinct  
171 (Rashid *et al.*, 2012; Table S2 & S3).

172

173 High-quality DNA was prepared using the method of Wilson (Wilson, 1987) with minor  
174 modifications (Cleenwerck *et al.*, 2002). DNA-DNA hybridizations were performed using  
175 a microplate method at 47.8 °C with photobiotin-labelled probes as described before  
176 (Goris *et al.*, 2007) using an HTS7000 Bio Assay Reader (PE Applied Biosystems) for  
177 fluorescence measurements. The DNA G+C content was determined by HPLC as  
178 described previously (Mesbah *et al.*, 1989). DDH experiments were conducted with the  
179 type strains of *R. etli* and *R. phaseoli*, since these two species were very close to the  
180 novel strains in phylogenetic analyses. The results of the DDH experiments are shown  
181 in Table 1. Strain BLR27<sup>T</sup> (proposed type strain of clade I) showed 50 % and 56 % DNA  
182 relatedness to the type strains of *R. etli* and to *R. phaseoli*, respectively. It showed 60 %  
183 relatedness to strain BLR175<sup>T</sup> (proposed type strain of clade II) and 50 % DNA  
184 relatedness to strain BLR195<sup>T</sup> (proposed type strain of clade III). The strain BLR175<sup>T</sup>  
185 showed 53 % relatedness to BLR195<sup>T</sup>. Overall, the proposed type strains (BLR27<sup>T</sup>,  
186 BLR175<sup>T</sup> and BLR195<sup>T</sup>) showed 50 – 62 % relatedness to their most closely related  
187 species and 50 – 60 % relatedness to each other. The DNA G+C content of the novel  
188 strains were 61.1 %, 60.9 % and 61.4 % for BLR27<sup>T</sup>, BLR175<sup>T</sup> and BLR195<sup>T</sup>,  
189 respectively. These values are within the range of the genus *Rhizobium* (Jordan, 1984).

190

191 ANI is the best approach to determine genetic relatedness between two genomes  
192 because this method evaluates a large number of genes in its calculation, including  
193 slowly and fast evolving genes, and thus minimizes the effect of variable evolutionary  
194 rates or horizontal gene transfer events (Konstantinidis & Tiedje, 2005). Genomic DNA  
195 was extracted from strains grown in TY medium (Beringer, 1974) using PowerSoil DNA  
196 isolation kits (MoBio, Carlsbad, CA), and then fragmented, barcoded, quantitated and  
197 run as part of a batch of eight genomes on a 318 chip on an Ion Torrent PGM using the  
198 manufacturer's recommended protocols (Thermo Fisher, Waltham, MA). Each genome  
199 was assembled using the Newbler GS De Novo assembler version 2.8 (Roche  
200 diagnostics) with default parameter values. ANI was calculated within the JSpecies  
201 software (Richter & Rosselló-Móra, 2009). The Nucleotide MUMmer algorithm  
202 (NUCmer) was used, with default parameter settings, to calculate the ANI by subtracting  
203 the similarity errors from the alignment length (Kurtz *et al.*, 2004; Richter & Rosselló-  
204 Móra, 2009). Genomes were compared with each other, with genome assemblies  
205 obtained using the same methods for *Rhizobium pisi* DSM30132<sup>T</sup> and *Rhizobium fabae*  
206 CCBAU33202<sup>T</sup> (unpublished), and with complete genome assemblies downloaded from  
207 NCBI for the following strains: *Rhizobium etli* CFN42<sup>T</sup> (GCA\_000092045), *Rhizobium*  
208 *phaseoli* CIAT652 (GCA\_000020265), *Rhizobium leguminosarum*3841  
209 (GCA\_000009265), *Rhizobium leguminosarum* WSM1325 (GCA\_000023185),  
210 *Rhizobium* sp. WSM2304 (GCA\_000021345). Ion Torrent sequencing yielded 155 Mbp,  
211 264 Mbp, 237 Mbp of sequence from BLR27<sup>T</sup>, BLR175<sup>T</sup> and BLR195<sup>T</sup>, respectively,  
212 corresponding to 27- to 49-fold coverage, so it can be expected that virtually all the  
213 genomic sequence is included. Assembly resulted in 140, 89 and 187 contigs (>100 bp)  
214 with N50 sizes of 229 kb, 286 kb and 173 kb, respectively. Pairwise ANI was calculated  
215 between these genomic sequences, and with other strains in the *R. leguminosarum*  
216 species complex for which genome data were available (Table 2). Each ANI was  
217 calculated in both directions, but the results never differed by more than 0.03%. ANI  
218 values ranged from 87.27% to 92.39%, with this highest value being between BLR195<sup>T</sup>  
219 and *R. phaseoli* CIAT652. All these values are well below 96%, which is an accepted  
220 value as the species boundary, equivalent to a DDH of 70% (Richter & Rosselló-Móra,



221 2009). Hence, each of the three BLR strains belongs to a distinct species, and these are  
222 different from all the closely related species described previously.

223

224 Different phenotypic characteristics i.e. colony size, growth on LB medium, acid-alkali  
225 production, tolerance to NaCl, temperature and pH, and antibiotic sensitivity were  
226 determined following the protocols described in Rashid *et al.*, (2012). To observe the  
227 host range of proposed species for nodule formation, cross inoculation tests were  
228 performed with pea (*Pisum sativum*) and lathyrus (*Lathyrus sativa*) (Rashid *et al.*, 2012).  
229 Randomly selected all strains from three species were availed to form nodules with both  
230 pea and lathyrus suggesting that proposed new species are ideal members of  
231 symbiovar *viciae*. Utilization of different carbon substrates by proposed species and  
232 close relatives were determined using Biolog GENIII following manufacturer's  
233 instructions and results are mentioned in Table S1. The cellular fatty acid compositions  
234 of type strain of proposed species were analyzed after growing on YEMA plates at 28  
235 °C for 3 days. Cells were saponified and transmethylated as described by Kuykendal *et*  
236 *al.* (1988) and were separated by using the Sherlock microbial identification system  
237 (RTSBA6; MIDI) and an Agilent (model 680N) gas chromatograph, and were  
238 determined at DSMZ, Germany. The results of fatty acid analysis are mentioned in  
239 Table S4. Phenotypic characteristics of strains belonging to the three proposed species  
240 and their close relatives are given in Table S5. *R. binae* and most *R. bangladeshense*  
241 strains were able to grow at pH 10, and also in 0.5 % NaCl, unlike most strains of *R.*  
242 *lentis*. On the other hand, all *R. lentis* and some *R. bangladeshense* strains showed  
243 resistance to ampicillin compared to their close relative (*R. etli*). The type strains of the  
244 proposed species and their close relatives shared few common fatty acids: 16:0; 18:0;  
245 16:0 3 OH; 19:0 cyclo ω8c; summed feature 2 and summed feature 8 but the amounts  
246 were different with close relatives and among themselves. Moreover, 15:0 iso 2 OH and  
247 summed feature 3 are found in two of the proposed species. Carbon and nitrogen  
248 substrates utilization pattern of proposed novel species differed with their close relatives  
249 and among themselves.

250

251 We have selected one representative strain from each clade that was identified in the  
252 housekeeping gene phylogeny, and demonstrate through the use of DDH and ANI  
253 analysis that they do indeed meet the standard criteria for distinct species, since all  
254 DDH values are below 70% and ANI values below 96%. DDH has been the standard  
255 method for bacterial species demarcation for the last 50 years (McCarthy &  
256 Bolton, 1963; Tindall *et al.*, 2010, Wayne *et al.*, 1987), but it has major limitations. It is  
257 time-consuming, laborious, and hard to standardize between laboratories. An  
258 increasingly significant limitation, as more species are described, is that DDH requires a  
259 laboratory comparison with all possible close relatives. DDH was developed before  
260 genome sequencing became feasible, but sequence-based methods have the potential  
261 to provide more reliable information more easily. The calculation of average nucleotide  
262 identity (ANI) from genome sequence data has been shown to give comparable results  
263 to DDH, with a species boundary at around 96% (Goris *et al.*, 2007; Konstantindis &  
264 Tiedje, 2005). Our study does not test the accuracy of this boundary, since all ANI  
265 values were much lower than this, but it does provide further evidence that low ANI  
266 values can be used as an effective substitute for DDH when establishing that strains do  
267 not belong to the same species. We expect that, in future, proposals for new bacterial  
268 species will increasingly use ANI evidence in place of DDH. In this study, the two  
269 methods provide consistent evidence that three new species are involved in lentil  
270 nodulation in Bangladesh, for which we proposed the names *Rhizobium lentis*,  
271 *Rhizobium bangladeshense* and *Rhizobium binae*.

272

### 273 **Description of *Rhizobium lentis***

274 *Rhizobium lentis* (len'tis. L. gen. n. lentis, referring to *Lens*, the plant genus from which  
275 the bacteria were isolated).

276 Cells are Gram negative, aerobic, non-spore forming and rod shaped. The optimum  
277 growth temperature is 28 °C at pH 7. Colonies are circular, convex and creamy white on  
278 YEMA medium. Strains grow at 12 – 37 °C but can survive at 4 °C. Strains grow well at  
279 pH 5.5 to 8.2 and are sensitive to 0.5 % NaCl in YEMA medium. Most of the strains are  
280 resistant to ampicillin, kanamycin and nalidixic acid. Strains do not tolerate tetracycline  
281 and do not show any growth on LB medium. The fatty acid composition of type strain is

282 15:0 iso 2-OH, 16:0, 16:0 3-OH, 18:0, 19:0 cyclo  $\omega$ 8c, summed featured 2, summed  
283 featured 3 and summed featured 8. In Biolog III systems, type strain could utilize  $\alpha$ -D  
284 lactose,  $\beta$ -methyl-D-glucoside, D-sorbitol, D-mannito,D-arbitol-glycerol, D-fructose-6-  
285 phosphate, L-aspartic acid, D-gluconic acid, mucic acid, D-lactic acid methyl ester, L-  
286 lactic acid, L-histidine, L-lactic acid,  $\beta$ -hydroxy-D, L-butyric acid, D-malic acid, L-malic  
287 acid, acetic acid and formic acid. Type strain was unable to utilize D-maltose, D-  
288 trehalose, D-cellobiose, gentiobiose, sucrose, D-raffinose,  $\alpha$ -D-glucose, D-turanose, D-  
289 melibiose, mannose, galactose, 3-methyle glucose, inosine, D-aspartic acid, glycyl-L-  
290 proline, L-alanine, L-aspartic acid, L-arginine, L-serine, pectine, D-saccharic acid, p-  
291 hydroxy-phenylacetic acid, methyl pyruvate, citric acid, bromo-succinic acid, acetoacetic  
292 acid or propionic acid. Type strain could grow in the presence of lincomycin, tetrazolium  
293 violet, tetrazolium blue and nalidixic acid, but not with 1% sodium lactate,  
294 troleandomycin, lithium chloride,potassium tellurite or sodium butyrate.

295  
296 Type strain is BLR27<sup>T</sup> (= LMG 28441<sup>T</sup> = DSMZ 29286<sup>T</sup>). The DNA G+C content of type  
297 strain is 61.1%. Type strain was isolated from effective nodules of *Lens culinaris* from  
298 Bagatipara, Natore district of Bangladesh. Other strains (BLR9, BLR26, BLR28, BLR29,  
299 BLR33, BLR41, BLR45, BLR59, BLR87, BLR98, BLR100, BLR122, BLR127, BLR137,  
300 BLR139 and BLR160) were isolated from different parts of Bangladesh.

### 301 **Description of *Rhizobium bangladeshense***

302 *Rhizobium bangladeshense*, (ban.gla.desh.en'se. N.L. neut. adj. *bangladeshense*, from  
303 Bangladesh, referring to the geographical origin of the strains).

304 Cells are Gram negative and rod shaped. Colonies are circular, convex and creamy  
305 white on YEMA medium. The optimum temperature for growth is 28 °C at pH 7,  
306 although strains could grow well up to 37 °C. Strains can tolerate pH values between  
307 5.5 and 10 and are sensitive to ampicillin, resistant to kanamycin and nalidixic acid, and  
308 grow well in YEMA medium containing 0.5 % NaCl. Strains do not tolerate tetracycline  
309 and do not show any growth on LB medium. Fatty acid composition of type strain is  
310 16:0, 16:0 3-OH, 18:0, 19:0 cyclo  $\omega$ 8c, summed featured 2 and summed featured 8. In  
311 Biolog III systems type strain could utilize D-maltose, D-trehalose, D-cellobiose,  
312 gentiobiose, sucrose, D-raffinose,  $\alpha$ -D-glucose, D-turanose,  $\alpha$ -D lactose, D-fructose,  $\beta$ -

313 methyl-D-glucoside, salicin, N-acetyl-D-galactosamine, D-sorbitol, D-mannitol, D-arbitol,  
314 glycerol, D-glucose-6-phosphate, D-gluconic acid, quinic acid, D-saccharic acid, D-lactic  
315 acid methyl ester, lactic acid,  $\alpha$ -keto-glutaric acid and tween 40. Type strain failed to  
316 utilize dextrin, D-aspartic acid, glycyl-L-proline, L-alanine, L-arginine, L-glutamic acid, L-  
317 histidine, L-serine, mucic acid, p-hydroxy-phenylacetic acid, methyl pyruvate, citric acid,  
318 D-malic acid, L-malic acid, propionic acid or formic acid. Type strain could grow in the  
319 presence of lincomycin and potassium tellurite, but not with 1% sodium lactate,  
320 troleandomycin, tetrazolium violet, tetrazolium blue, Nalidixic acid, lithium chloride and  
321 sodium butyrate.

322 Type strain is BLR175<sup>T</sup> (= LMG 28442<sup>T</sup> = DSMZ 29287<sup>T</sup>). The DNA G+C content of type  
323 strain is 61 %. Type strain was isolated from effective nodules of *Lens culinaris* from  
324 Mohammedpur, Khulna district of Bangladesh. Other strains (BLR62, BLR99, BLR129,  
325 BLR153 and BLR154) were isolated from different parts of Bangladesh.

#### 326 **Description of *Rhizobium binae***

327 *Rhizobium binae* (bi'nae. N.L. gen. fem. n. binae, abbreviation for Bangladesh Institute  
328 of Nuclear Agriculture, a research institute where the first steps to isolate the bacteria  
329 were taken).

330 Cells are Gram negative and rod shaped. Colonies are circular, convex and creamy  
331 white on YEMA medium. The optimum temperature for growth is 28 °C at pH 7, but the  
332 strains grow well at 37 °C. Strains survive at pH values between 5.5 and 10 and tolerate  
333 1 % NaCl in YEMA. They are very sensitive to ampicillin and resistant to kanamycin and  
334 nalidixic acid. Strains do not tolerate tetracycline and do not show any growth on LB  
335 medium. Fatty acid composition of type strain is 15:0 iso 2-OH, 16:0, 16:0 3-OH, 18:0,  
336 18:1 $\omega$ 9c, 18:0 3-OH, 18:1 $\omega$ 7c 11methyl, 19:0 cyclo  $\omega$ 8c, summed feature 2 and  
337 summed feature 8. Type strain could utilize dextrin, D-maltose, D-trehalose, D-  
338 cellobiose, gentiobiose, sucrose, D-raffinose,  $\alpha$ -D-glucose, D-turanose,  $\alpha$ -D lactose, D-  
339 fructose, D-melibiose,  $\beta$ -methyl-D-glucoside, salicin, N-acetyl-D-galactosamine, D-  
340 mannose, D-galactose, D-mannitol, D-sorbitol, D-arabitol, glycerol, D-glucose-6-  
341 phosphate, D-fructose-6-phosphate, D-alanine, L-aspartic acid, L-histidine, L-  
342 pyroglutamic acid, quinic acid, D-saccharic acid, methyl pyruvate, L-lactic acid, citric  
343 acid, D-malic acid, L-malic acid, bromo-succinic acid,  $\beta$ -hydroxy-d,l-butyrac acid and

344 acetic acid. Type strain failed to utilize N-acetylc-D-mannosamine, 3-methyl glucose,  
345 inosine, glycyl-L-proline, L-arginine, D-galacturonic acid, D-glucuronic acid,  
346 glucuronamide, p-hydroxy-phenylacetic acid, D-lactic acid methyl ester,  $\alpha$ -keto-glutaric  
347 acid, tween 40, propionic acid or formic acid. Type strain could grow in the presence of  
348 lincomycin and potassium tellurite but not with 1% sodium lactate, troleandomycin,  
349 lithium chloride or sodium butyrate.

350

351 Type strain is BLR195<sup>T</sup> (=LMG 28443<sup>T</sup> = DSM 29288<sup>T</sup>). The DNA G+C content of type  
352 strain is 61.5 %. Type strain was isolated from effective nodules of *Lens culinaris* from  
353 Sarishadi, Feni district of Bangladesh. All strains (BLR 228, BLR235 and the type strain)  
354 were isolated from the Southeast part of Bangladesh.

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362

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542 **Fig. 1.** ML tree from 16S rRNA gene partial sequences. Bootstrap values  
543  $\geq 70$  are indicated for each node (1000 replicates).  
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559 **Fig. 2.** ML tree from concatenated sequence of atpD-recA genes. Bootstrap  
560 values  $\geq 70$  are indicated for each node (1000 replicates).

561 **Tables**

562 **Table 1.** Genetic relatedness measured by DDH between strains representing the novel clades and type strains of the  
 563 most closely related species

Species	Strain	DNA G+C content (% mol)	DNA-DNA relatedness (%)				
			<i>Rhizobium lentis</i> (BLR27 <sup>T</sup> )	<i>Rhizobium bangladeshense</i> (BLR175 <sup>T</sup> )	<i>Rhizobium binae</i> (BLR195 <sup>T</sup> )	<i>Rhizobium etli</i> (LMG 17827 <sup>T</sup> )	<i>Rhizobium phaseoli</i> (LMG 8819 <sup>T</sup> )
<i>Rhizobium lentis</i>	BLR 27 <sup>T</sup>	61.1	100.0	60.9	50.4	50.6	56.3
<i>Rhizobium bangladeshense</i>	BLR175 <sup>T</sup>	61.0	60.9	100.0	53.2	49.2	62.2
<i>Rhizobium binae</i>	BLR195 <sup>T</sup>	61.5	50.4	53.2	100.0	54.9	55.8

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568 **Table 2.** Average nucleotide identity (ANI) between strains representing the novel clades and the most closely related  
 569 sequenced members of the *Rhizobium leguminosarum* complex

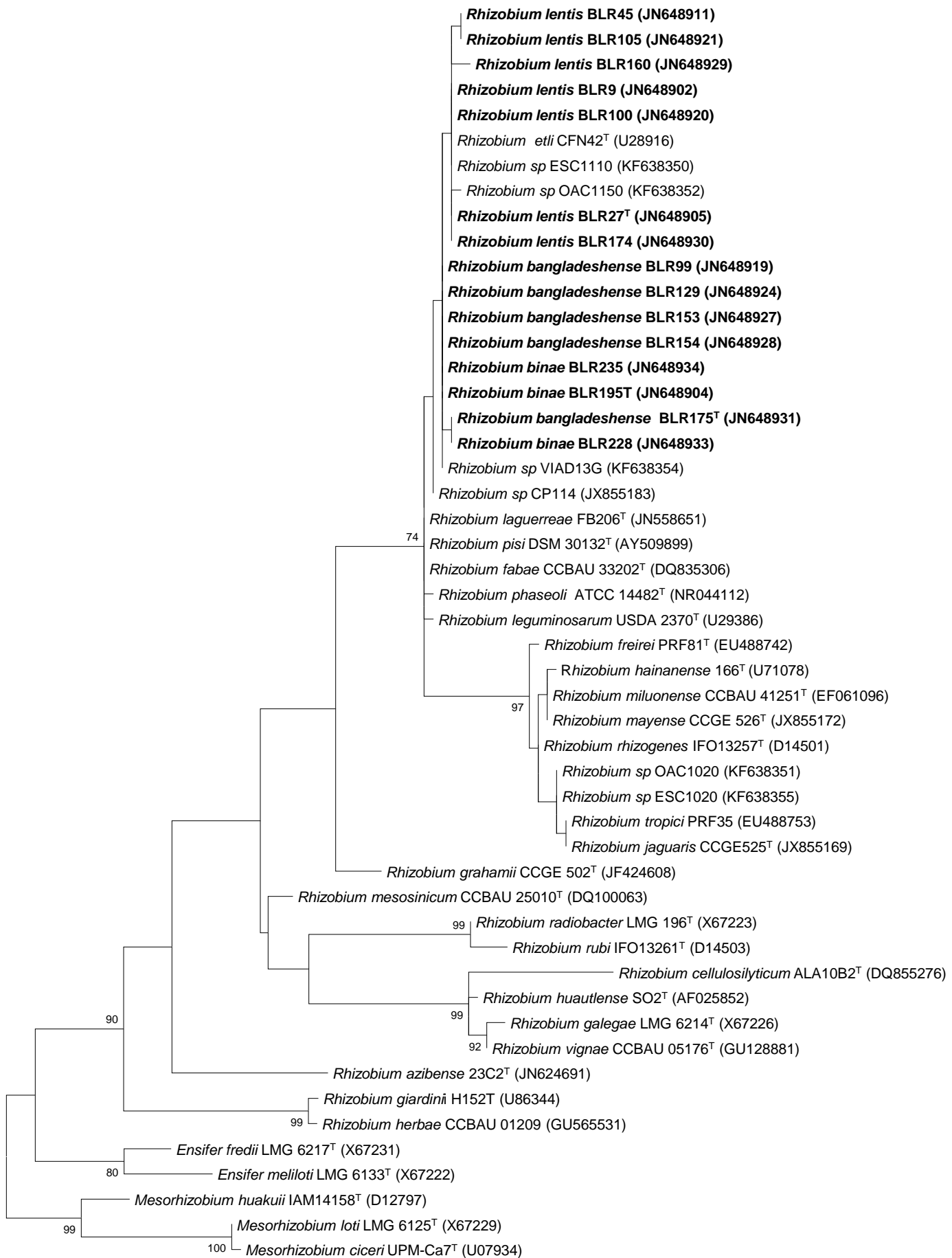
Species	Strain	Average Nucleotide Identity (%)		
		<i>R. lentis</i> BLR 27 <sup>T</sup>	<i>R. bangladeshense</i> BLR 175 <sup>T</sup>	<i>R. binae</i> BLR 195 <sup>T</sup>
<i>R. lentis</i>	BLR27 <sup>T</sup>	100	89.70	88.64
<i>R. bangladeshense</i>	BLR175 <sup>T</sup>	89.72	100	88.51
<i>R. binae</i>	BLR195 <sup>T</sup>	88.62	88.51	100
<i>R. etli</i>	CFN 42 <sup>T</sup>	89.04	88.64	89.28
<i>R. phaseoli</i>	CIAT 652	88.56	88.25	92.39
<i>R. fabae</i>	CCBAU 33202 <sup>T</sup>	88.90	88.64	88.81
<i>R. pisi</i>	DSM 30132 <sup>T</sup>	88.82	88.18	88.46
<i>R. leguminosarum</i>	3841	88.33	87.27	88.28
<i>R. leguminosarum</i>	WSM1325	88.01	87.59	88.06
<i>Rhizobium</i> sp.	WSM2304	88.09	87.76	88.55

570 Abbreviation: *R.* = *Rhizobium*

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**Fig. 1.**

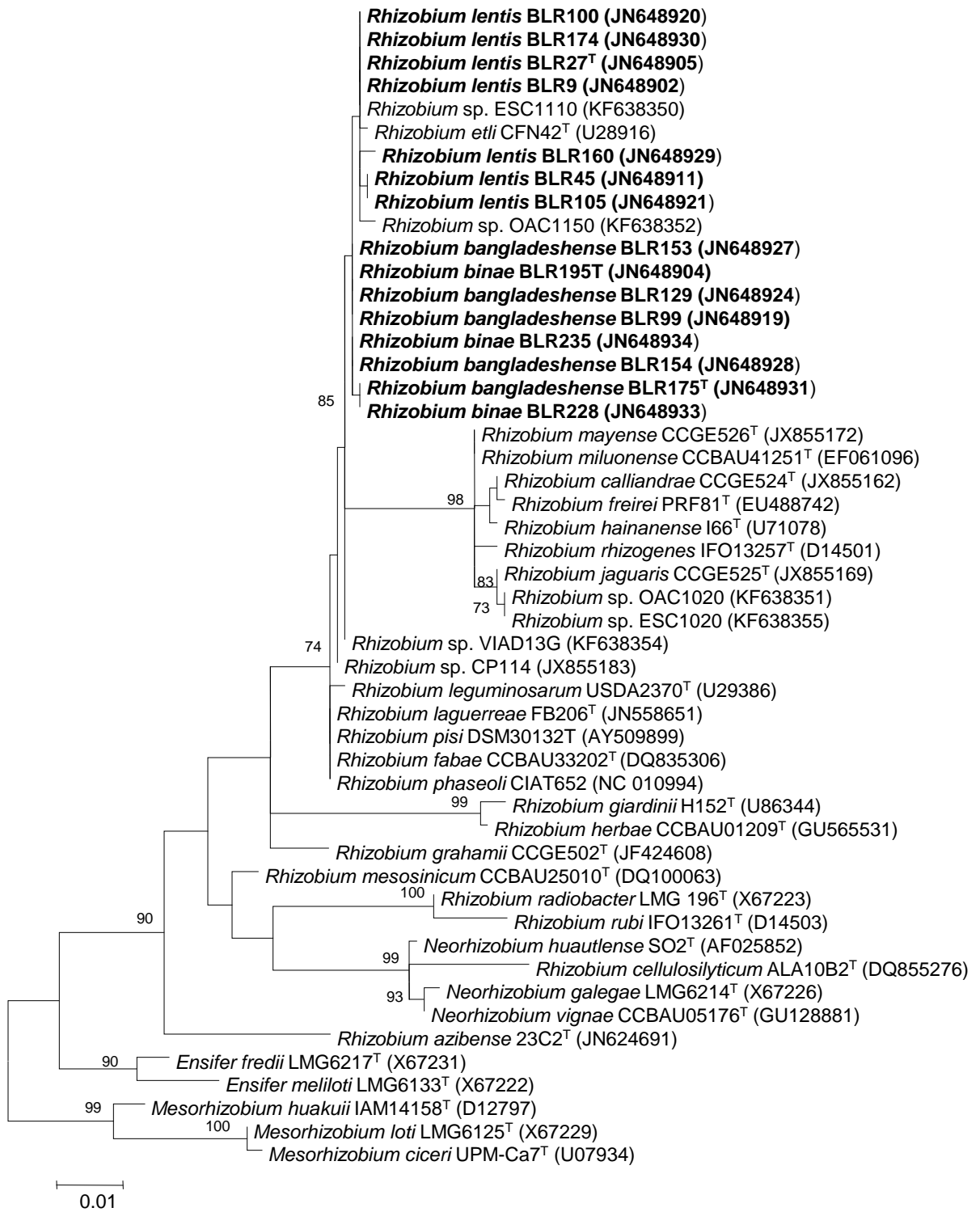
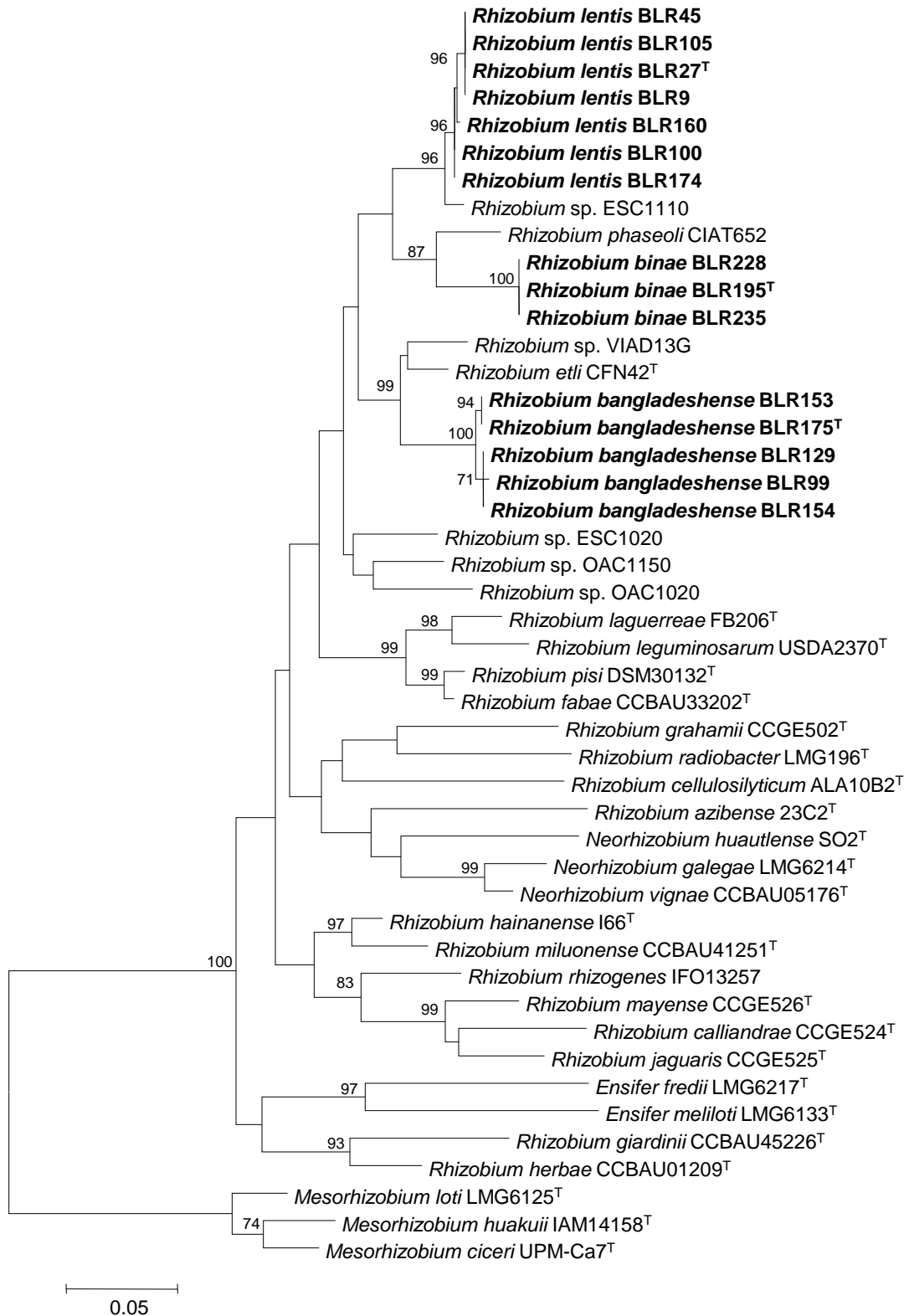




Fig. 2.



## Figure legends

**Fig. 1.** ML tree from 16S rRNA gene partial sequences. Bootstrap values  $\geq 70$  are indicated for each node (1000 replicates).

**Fig. 2.** ML tree from concatenated sequence of atpD-recA genes. Bootstrap values  $\geq 70$  are indicated for each node (1000 replicates).

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2 Average nucleotide identity of genome sequences supports the description of *Rhizobium lentis* sp. nov.,  
3 *Rhizobium bangladeshense* sp. nov. and *Rhizobium binae* sp. nov. from lentil (*Lens culinaris*) nodules

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24 **Table S1.** Genetic distances of type strains between proposed species and their close species

25

Species	Strains	Genetic distance (%)*					
		<i>Rhizobium lentis</i>		<i>Rhizobium bangladeshense</i>		<i>Rhizobium binae</i>	
		<i>atpD</i>	<i>recA</i>	<i>atpD</i>	<i>recA</i>	<i>atpD</i>	<i>recA</i>
<i>Rhizobium lentis</i>	BLR27 <sup>1</sup>	0.0	0.0	6.2	9.3	6.2	8.3
<i>Rhizobium bangladeshense</i>	BLR175 <sup>T</sup>	6.2	9.3	0.0	0.0	6.4	11.1
<i>Rhizobium binae</i>	BLR195 <sup>1</sup>	6.2	8.3	6.4	11.1	0.0	0.0
<i>Rhizobium etli</i>	CFN42 <sup>T</sup>	5.4	8.7	5.6	3.8	6.2	11.2
<i>Rhizobium phaseoli</i>	CIAT652	5.6	7.2	6.4	11.0	5.4	5.2
<i>Rhizobium fabae</i>	CCBAU33202 <sup>1</sup>	9.3	7.9	8.6	9.2	8.6	8.9
<i>Rhizobium pisi</i>	DSM30132 <sup>T</sup>	9.5	8.4	8.7	9.1	8.6	9.4
<i>Rhizobium leguminosarum</i>	USDA 2370 <sup>T</sup>	10.6	7.9	11.4	9.7	11.3	8.9

26 \* "Genetic distance (%)\*": percentage of non-identical nucleotides"

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33 **Table S2.** Genetic distances among the strains within species

Species	Genetic distances (%)*	
	Genes	
	<i>atpD</i>	<i>recA</i>
<i>Rhizobium lentis</i> (7 strains)	1.0	0.0
<i>Rhizobium bangladeshense</i> (5 strains)	0.4	0.5
<i>Rhizobium binae</i> (3 strains)	0.1	0.0

34 \* "Genetic distance (%)" : percentage of non-identical nucleotides"

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48 **Table S3.** Additional differential substrate utilization patterns of the new species and closely related species. Data were obtained using Biolog  
 49 GENIII systems according to the manufacturer's instructions. +, positive reaction, w, weak positive reaction, - negative reaction

	<i>R.</i> <i>bangladeshense</i> BLR 175 <sup>T</sup>	<i>R. binae</i> BLR 195 <sup>T</sup>	<i>R. lentis</i> BLR 27 <sup>T</sup>	<i>R. etli</i> LMG 17827T	<i>R. fabae</i> LMG 23997 <sup>T</sup>	<i>R.</i> <i>laguerreae</i> LMG 27434 <sup>1</sup>	<i>R.</i> <i>leguminosarum</i> LMG 14904 <sup>1</sup>	<i>R. phaseoli</i> LMG 8819 <sup>1</sup>
<b>Growth on:</b>								
Dextrin	-	+	-	-	w	+	+	+
D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-raffinose, α-D- glucose	+	+	-	-	+	+	-	+
D-turanose	+	+	-	-	+	+	-	-
α-D-lactose, D-fructose	+	+	+	-	+	+	+	+
D-melibiose	w	+	-	w	+	+	+	+
β-methyl-D-glucoside, salicin	+	+	+	-	+	+	+	+
N-acetyl-D-mannosamine	w	-	-	-	-	+	+	-
N-acetyl-D-galactosamine	+	+	w	-	w	+	+	+
D-mannose	w	+	-	-	+	+	-	+
D-galactose	w	+	-	-	+	+	+	+
3-Methyl glucose	w	-	-	-	-	-	+	-
Inosine	w	-	-	-	-	-	-	+
D-sorbitol	+	+	+	-	+	+	-	+
D-mannitol	+	+	+	w	+	+	-	+
D-arabitol, glycerol	+	+	+	-	+	+	+	+
D-glucose-6-phosphate	+	+	w	w	-	-	+	+
D-fructose-6-phosphate	w	+	+	w	-	+	+	+
D-aspartic acid	-	w	-	-	-	+	-	+
Glycyl-L-proline	-	-	-	-	-	+	-	-
L-alanine	-	+	-	-	+	-	-	+
L-arginine	-	-	-	-	+	+	w	+

	<i>R. bangladeshense</i> BLR 175 <sup>T</sup>	<i>R. binae</i> BLR 195 <sup>T</sup>	<i>R. lentis</i> BLR 27 <sup>T</sup>	<i>R. etli</i> LMG 17827T	<i>R. fabae</i> LMG 23997 <sup>T</sup>	<i>R. laguerreae</i> LMG 27434 <sup>T</sup>	<i>R. leguminosarum</i> LMG 14904 <sup>T</sup>	<i>R. phaseoli</i> LMG 8819 <sup>T</sup>
L-aspartic acid	w	+	+	-	+	+	-	+
L-glutamic acid	-	w	-	-	+	+	+	+
L-histidine	-	+	w	-	+	+	+	w
L-pyroglutamic acid	w	+	w	-	-	+	-	+
L-serine	-	w	-	-	-	+	-	+
Pectin	w	w	-	-	-	+	-	+
D-galacturonic acid	w	-	-	-	-	+	-	-
D-gluconic acid	+	w	+	-	-	+	+	+
D-glucuronic acid	w	-	w	w	-	+	-	w
Glucuronamide	w	-	w	+	w	+	+	w
Mucic acid	-	w	+	-	w	+	+	+
Quinic acid	+	+	w	-	+	+	w	+
D-Saccharic acid	+	+	-	-	-	+	-	+
p-Hydroxy-phenylacetic acid	-	-	-	-	-	+	-	-
Methyl pyruvate	-	+	-	-	+	+	-	-
D-lactic acid methyl ester	+	-	+	-	-	+	-	-
L-lactic acid	+	+	+	-	-	+	+	-
citric acid	-	+	-	-	+	+	+	-
α-keto-glutaric acid	+	-	w	w	-	+	+	-
D-malic acid, L-malic acid	-	+	+	-	+	+	+	+
Bromo-succinic acid	w	+	-	-	+	+	-	+
Tween 40	+	-	w	-	-	+	w	-
α-Hydroxy-butyric acid	w	w	w	-	w	+	w	-
β-Hydroxy-D,L-butyric acid	w	+	+	-	w	+	w	w
Acetoacetic acid	w	w	-	-	+	+	-	-
Propionic acid	-	-	-	-	+	+	+	-
Acetic acid	w	+	+	-	+	+	+	+

	<i>R. bangladeshense</i> BLR 175 <sup>T</sup>	<i>R. binae</i> BLR 195 <sup>T</sup>	<i>R. lentis</i> BLR 27 <sup>T</sup>	<i>R. etli</i> LMG 17827 <sup>T</sup>	<i>R. fabae</i> LMG 23997 <sup>T</sup>	<i>R. laguerreae</i> LMG 27434 <sup>T</sup>	<i>R. leguminosarum</i> LMG 14904 <sup>T</sup>	<i>R. phaseoli</i> LMG 8819 <sup>T</sup>
Formic acid	-	-	+	-	+	+	w	-
<b>Growth at pH 6</b>	-	+	-	-	w	+	-	-
<b>Growth in the presence of:</b>								
1% NaCl	-	-	-	-	w	+	-	-
1% Sodium lactate	-	-	-	-	-	+	-	-
Troleandomycin	-	-	-	-	-	+	+	-
Rifamicin SV	w	+	+	-	+	+	+	+
Lincomycin	+	+	+	+	+	+	+	-
Tetrazolium violet	-	w	+	+	w	-	+	+
Tetrazolium blue	-	w	+	+	-	-	+	+
Nalidixic acid	-	+	+	+	+	+	+	w
Lithium chloride	-	-	-	-	-	+	-	-
Pottasium tellurite	+	+	-	+	+	+	+	+
Sodium butyrate	-	-	-	+	-	-	-	-

50 In Biolog III systems, all strains were able to grow on D-fucose, L-fucose and L-rhamnose and none of the strains was able to use D-serine, D-  
51 galactonic acid lactone or  $\alpha$ -keto-butyric acid. None of the strains could grow at pH 5 or in the presence of 4% or 8% NaCl, fusidic acid, D-serine,  
52 minocycline, guanidine HCl, niaproof 4, vancomycin, aztreonam or sodium bromate in Biolog III systems.

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59 **Table S4.** Fatty acid pattern of type strain of proposed three new species.

Fatty acid	<i>Rhizobium lentis</i> (BLR27 <sup>1</sup> )	<i>Rhizobium bangladeshense</i> (BLR175 <sup>1</sup> )	<i>Rhizobium binae</i> (BLR195 <sup>1</sup> )
15:0 iso 2 OH	0.55	absent	0.79
16:0	2.03	2.67	2.12
16:0 3 OH	0.91	2.16	0.80
18:0	3.23	6.88	4.63
18:1 $\omega$ 9c	absent	absent	2.36
18:0 3 OH	absent	absent	0.97
18:1 $\omega$ 7c 11methyl	absent	absent	0.63
19:0 cyclo $\omega$ 8c	11.37	6.66	21.76
*Summed featured 2	5.80	7.00	5.62
†Summed featured 3	0.43	absent	absent
‡Summed featured 8	75.35	74.62	60.32

60 Summed featured indicate two or more fatty acids that could not be separated by MIDI system.

61 \*Summed featured 2 consist one or more of 12:0 (aldehyde), unknown ECL 15.489, 14:0 3 OH / 16:1 iso I.

62 †Summed featured 3 consists 16:1 $\omega$ 7c / 16:1 $\omega$ 6c.

63 ‡Summed featured 8 consists 18:1 $\omega$ 7c / 18:1 $\omega$ 6c

64 NB: Fatty acids present in <0.5% are not mentioned in this table.

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77 **Table S5.** Phenotypic characteristics of proposed three new species and their close relatives

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Species	NaCl tolerance (%)			Temperature tolerance ( °C)	pH tolerance		Antibiotic sensitivity ( $\mu\text{g mL}^{-1}$ )						
	0.5	1.0	2.0	37	8.2	10	Ampicillin					Kanamycin	
							50	75	100	125	150	10	20
<i>R. lentis</i> (21 strains)	—	—	—	+	+	v	+	+	+	v	v	+	+
<i>R. bangladeshense</i> (6 strains)	+	—	—	+	+	+	+	v	v	v	v	+	+
<i>R. binae</i> (3 strains)	+	v	—	+	+	+	+	—	—	—	—	+	v
<i>R. etli</i> *	—	—	ND	+	—	—	—	—	—	—	—	—	—
<i>R. phaseoli</i> *	+	—	—	v	—	—	+	+	+	+	+	ND	ND
<i>R. pisi</i> *	+	+	—	+	+	—	—	—	—	—	—	ND	ND
<i>R. fabae</i> *	+	+	+	+	+	—	+	+	+	+	+	+	+
<i>R. leguminosarum</i> *	+	—	—	—	+	ND	v	v	v	v	v	ND	ND
<i>R. laguerreae</i> *	+	—	—	+	ND	ND	v	v	v	v	v	ND	ND

79 Abbreviations: + = growth positive for >80% of strains, — = growth negative for >80% of strains, v = strains varied in response and w= weak response. ND = not determined / data  
80 not available, *R.* = *Rhizobium*.

81 \* Data taken from Rashid *et al.* (2012) and published species descriptions (Ramírez-Bahena *et al.*, (2008); Saïdi *et al.*, (2014); Segovia *et al.*, (1993).

82 N.B.: All strains grew at 32°C and showed acidic reaction on BTB medium, but none grew at 40°C temperature, in tetracycline (5  $\mu\text{g mL}^{-1}$ ) or on LB medium.

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