

1 **A promiscuity locus confers *Lotus burttii* nodulation with rhizobia from five different**
2 **genera**

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21

22 **Abstract**

23 Legumes acquire access to atmospheric nitrogen through nitrogen fixation by rhizobia in root
24 nodules. Rhizobia are soil dwelling organisms and there is a tremendous diversity of rhizobial
25 species in different habitats. From the legume perspective, host range is a compromise
26 between the ability to colonize new habitats, where the preferred symbiotic partner may be
27 absent, and guarding against infection by suboptimal nitrogen fixers. Here, we investigate
28 natural variation in rhizobial host range across *Lotus* species. We find that *Lotus burtii* is
29 considerably more promiscuous than *Lotus japonicus*, represented by the Gifu accession, in
30 its interactions with rhizobia. This promiscuity allows *Lotus burtii* to form nodules with
31 *Mesorhizobium*, *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, and *Allorhizobium* species that
32 represent five distinct genera. Using recombinant inbred lines, we have mapped the
33 Gifu/*burtii* promiscuity QTL to the same genetic locus regardless of rhizobial genus,
34 suggesting a general genetic mechanism for host-range expansion. The Gifu/*burtii* QTL now
35 provides an opportunity for genetic and mechanistic understanding of promiscuous legume-
36 rhizobia interactions.

37

38 **Keywords:** symbiotic nitrogen fixation, *Lotus*, host range, genetic mapping, rhizobia

39

40 **Introduction**

41 Symbiosis with nitrogen-fixing rhizobia in root nodules enable legumes to access
42 atmospheric nitrogen. In most cases, rhizobial entry into root cells requires recognition of
43 rhizobial Nodulation factors (NF) signalling molecules that are secreted in response to
44 perception of plant flavonoids (Oldroyd 2013). In turn, host membrane-bound Nod factor
45 receptors (NFRs) initiate downstream signal transduction pathways initiating rhizobial
46 infection and nodule organogenesis (Madsen et al. 2003; Radutoiu et al. 2003; Murakami et
47 al. 2018). Plants produce complex mixtures of flavonoids (Liu and Murray 2016). Likewise,
48 rhizobia secrete many different Nod factor species (D’Haeze and Holsters 2002), and both
49 flavonoid and Nod factor pools may change dynamically over time within the same plant

50 accession or rhizobia strain (Liu and Murray 2016; Kelly et al. 2018). This provides an
51 intricate system that, along with bacterial effectors and exopolysaccharides and the
52 corresponding plant detection systems, allows fine tuning of legume-rhizobium compatibility
53 (Yang et al. 2010; Kawaharada et al. 2015; Kusakabe et al. 2020). The successful
54 establishment of nitrogen fixation requires full compatibility of symbiotic partners.
55 Symbiotic compatibility can affect early stages of infection, determining whether or not
56 nodules are formed, or later stages of nodule development, affecting nitrogen-fixing
57 efficiency or nodule senescence (Perret et al. 2000; Wang et al. 2012; Yang et al. 2017).

58 Intercellular infection is thought to represent a more ancient and less advanced infection
59 mode than intracellular infection through root hairs (Sprent 2007). Specific legumes are
60 typically infected either intra- or intercellularly. However, at least some legumes maintain
61 genetic programs for both types of infection. These include *Sesbania rostrata*, where the
62 infection mode can change in response to flooding (Herder et al. 2006), and *Lotus* species,
63 where intercellular infection appears to serve as a backup function to the preferred
64 intracellular infection route through root hair infection threads. This phenomenon was
65 observed in *Lotus japonicus* Gifu as rare infection events of spontaneous nodules in a NF
66 receptor deficient genetic background (Madsen et al. 2010) and has since been found in the
67 *L. japonicus* Gifu interaction with IRBG74 (Montiel et al. 2020) and in *Lotus burtii*
68 interactions with *Sinorhizobium fredii* HH103 and *Rhizobium leguminosarum* Norway
69 (Acosta-Jurado et al. 2016b; Liang et al. 2019). Generally, intracellular root hair infection
70 appears to offer more stringent scrutiny of the rhizobial partner, whereas the compatibility
71 requirements for intercellular crack entry appears to be more relaxed (Sprent 2007; Madsen
72 et al. 2010).

73 In *Lotus*, both inter- and intracellular infection depend on NF signalling (Acosta-Jurado et al.
74 2016b; Montiel et al. 2020), except in rare cases, if organogenesis is activated in the absence
75 of Nod factor signalling (Madsen et al. 2010). Candidate gene approaches relying on
76 interspecific variation in Nod factor receptors has been used to demonstrate their roles in
77 determining compatibility (Radutoiu et al. 2007). Effectors and secretion system components
78 that deliver effectors into plant cells also affect compatibility. For instance, the
79 bradyrhizobial NopP effector is recognised by soybeans carrying the *Rj2* NLR resistance

80 gene, leading to termination of infection (Sugawara et al. 2018), and the *Bradyrhizobium*
81 *elkanii* NopF effector prevents infection in *Lotus japonicus*, but not in *L. burtii* (Kusakabe
82 et al. 2020). Rhizobial effectors can also promote symbiotic interactions as exemplified by
83 the *B. elkanii* effector Bel2-5, which confers the ability to nodulate soybeans deficient in Nod
84 factor perception (Ratu et al. 2021). Rhizobial genes that affect Nod factor and
85 exopolysaccharide production can also influence host range as demonstrated by the
86 *Sinorhizobium fredii* HH1103 *mucR1*, *syrM*, *nolR* and *nodD2* mutants (Acosta-Jurado et al.
87 2016a). Remarkably, *syrM*, *nolR* and *nodD2* mutations induce a shift from inter- to
88 intracellular infection in *L. burtii* and extend the host range to include *L. japonicus* Gifu
89 (Acosta-Jurado et al. 2019, 2020). In addition, *Lotus* intraspecific variation was exploited to
90 identify *Pxy* as a regulator of symbiotic compatibility downstream of exopolysaccharide
91 signalling (Kawaharada et al. 2021). Also in *Lotus*, *S. fredii* HH103 is able to form functional
92 nodules on *L. burtii*, whereas it induced ineffective nodules on *L. japonicus* Gifu, and a QTL
93 was mapped to chromosome 1 near the *Nfr1* gene (Sandal et al. 2012). Likewise, *L. burtii*
94 was also more permissive than *L. japonicus* Gifu in the interaction with *R. leguminosarum*
95 Norway (Grossmann et al. 2012).

96 Here, we investigate natural variation in rhizobial host range between the two *Lotus* species
97 *L. japonicus* Gifu and *L. burtii*, focusing on host control of symbiotic compatibility.

98

99 **Materials and Methods**

100

101 **Nodulation phenotyping**

102 For germination, seeds were scarified and surface sterilized with 0.5% sodium hypochlorite
103 for 15 minutes and rinsed several times with distilled water. Seeds were kept in sterile water
104 for 1 hour before sowing on wet filter paper. The germinated plants were transferred and
105 grown on 1/4 Broughton and Dilworth (B&D) medium (Broughton and Dilworth 1971)
106 where the surface of the agar slope was covered with filter paper. Rhizobia were grown in
107 yeast agar medium (YAM) except for *R. leguminosarum* strains that were grown in tryptone

108 yeast medium (TY). The strains were diluted to an OD₆₀₀ of 0.02 before inoculating with 50
109 µl per plant by pipetting the suspension directly on the root. The nodulation phenotype was
110 recorded at 35 days post-inoculation.

111

112 **QTL Analysis**

113 We used recombinant *L. japonicus* Gifu x *L. burttii* recombinant inbred lines (RILs) (Sandal
114 et al. 2012; Shah et al. 2016). For rough mapping, 18 RILs with balanced genotypes were
115 chosen (**Supplemental dataset 1**). Genotype and phenotype data were imported into R/qtI
116 version 3.4.2 (R Project for Statistical Computing, www.r-project.org/) using the read.cross
117 command. After converting to RIL format, the genetic map and missing genotype values
118 were estimated using est.map and mqmaugment, respectively. Multiple QTL Mapping
119 (MQM) was then conducted using 1000 permutations to determine significance thresholds.

120

121 **Hairy root transformation with *Nfr1* constructs**

122 The *L. burttii* *Nfr1* (BinG1) construct was based on the *L. japonicus* *Nfr1* complementation
123 construct, (carrying the entire *LjNfr1* gene driven by its own promoter), which was modified
124 using standard cloning techniques and transferred into the pIV10 integration vector
125 (AM235368). The construct was integrated into the *Agrobacterium rhizogenes* strain AR12
126 (Hansen et al. 1989). BinG1 was constructed as follows: a DNA fragment from position 4090
127 to position 4993 of the *Nfr1* gene (AJ575246/AJ575247) was substituted by the
128 corresponding fragment from *L. burttii* produced by PCR. The two *L. japonicus* Gifu/*L.*
129 *burttii* polymorphisms identified in the *Nfr1* extracellular region are both contained within
130 the *L. burttii* fragment included in the *BinG1* construct.

131

132 **Genotyping and phenotyping of F1 progeny of *L. burttii* and *L. japonicus* crosses**

133 Crosses using *L. burttii* as mother and *L. japonicus* Gifu as father, or the converse, were
134 generated. Seeds of *L. burttii* B-303, *L. japonicus* Gifu B-129 and the F1 progeny were
135 scarified and surface-sterilized as described earlier. Six-day old seedlings were transferred
136 into tulip shaped Weck jars (Weck 745) containing 300 ml sterilized sand-vermiculite
137 mixture supplemented with 40 ml of FAB medium. After two days, each plant was inoculated
138 with 1 ml of a *R. leguminosarum* Norway suspension ($OD_{600} = 0.005$) or 1 ml FAB medium
139 as a mock control. Plants were grown under a long-day photoperiod for six weeks and
140 phenotyped using a MZ16 FA stereomicroscope (Leica). For genotyping, genomic DNA was
141 extracted from leaves lysed in liquid nitrogen. Lysates were suspended in 500 μ l extraction
142 buffer (2% w/v CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH8) supplemented
143 with 3.1 μ l beta-mercaptoethanol and incubated at 65 °C for 20 min. Suspensions were mixed
144 with 300 μ l chloroform and centrifuged at 14,000 rpm for 5 min. Supernatants were mixed
145 with 1/10 volume of 3 M NaOAc and centrifuged at 14,000 rpm for 15 min. Pellets were
146 washed two times with 70% ethanol, air-dried and re-suspend in 50 μ l distilled water. The
147 DNA was used as template in PCR reactions with the *Lotus* marker TM1203 (forward:
148 TTGAATAAGGCTCATAGATCC, reverse: CTCAGTTTGGGTTTCAAGC) (Sato et al.,
149 2001) and verified by agarose gel electrophoresis.

150

151 **Results**

152

153 ***L. burttii* nodulates with rhizobia from five different genera**

154 It was previously reported that *S. fredii* HH103 forms functional nodules on *L. burttii* but not
155 on *L. japonicus* Gifu (Sandal et al. 2012). In order to determine if *L. burttii* is generally more
156 permissive in its symbiotic interactions than Gifu, we examined the nodulation phenotypes
157 of Gifu and *L. burttii* with a wide range of rhizobia from different genera including
158 *Sinorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Allorhizobium* and
159 *Mesorhizobium* (**Figure 1**). These distantly related rhizobial species produce NF with
160 different chemical modifications at the nonreducing and reducing ends (D’Haeze and

161 Holsters 2002; Beck et al. 2010; Renier et al. 2011) (**Table 1**;). We observed large variation
162 in nodule numbers and structures, which included nodule primordia “bumps”, white nodules
163 and in some cases small or more developed pink nodules, indicative of nitrogen fixation
164 (**Figure 2 A-D**). Among the 42 strains tested, only the cognate *Lotus* symbiont *M. loti* R7A
165 induced development of pink nodules in both Gifu and *L. burtii* (**Figure 2A and D**). At the
166 other extreme, *Sinorhizobium meliloti* nodulated neither. *Sinorhizobium fredii* NGR234,
167 which is compatible with a very broad range of legumes (Pueppke 1999), was unique in
168 promoting a larger number of pink nodules in Gifu than in *L. burtii* (**Figure 2D**;
169 **Supplementary Figure S1**). None of the remaining 39 strains formed pink nodules with
170 Gifu. In contrast, 30 out of the 39 strains formed at least some pink nodules with *L. burtii*
171 (**Figure 2A and D**). The 30 strains that nodulate *L. burtii* comprise five of the genera tested,
172 indicating that the host range of *L. burtii* is broad and that *L. burtii* is considerably more
173 promiscuous in its symbiotic interactions than Gifu, regardless the diverse composition of
174 the NF produced by the rhizobial strains (**Table 1**).

175

176 ***L. burtii* allows nodulation with inefficient nitrogen fixers**

177 Efficient nitrogen fixation by rhizobia in fully developed nodules has a positive impact on
178 the plant host, reflected in a more vigorous growth of the aerial part (Lindstrom and Mousavi,
179 2019). Accordingly, both Gifu and *L. burtii* plants nodulated by *M. loti* R7A showed a
180 significantly greater shoot length compared to mock-treated plants (**Figure 2A and E**). None
181 of the 41 remaining Gifu-rhizobial associations had a positive impact on shoot length. In
182 contrast, *L. burtii* growth was significantly enhanced by 10 different rhizobial species, nine
183 of which developed pink nodules. However, *R. leguminosarum* strain USDA2356 (strain
184 #33), which did not induce any pink nodules, resulted in significantly longer shoot lengths
185 than the mock control (**Figure 2A and E**), suggesting a growth promoting effect independent
186 of nitrogen fixation.

187 Despite formation of pink nodules, seventeen *L. burtii*-rhizobia interactions resulted in
188 plants displaying similar shoot length to mock-treated plants, whereas a significant reduction
189 of plant growth was observed in plants nodulated by nine rhizobial species (**Figure 2A and**

190 E). These results show that although a wide range of rhizobial species developed pink
191 nodules with *L. burttii*, most of them did not have a positive impact on growth and instead
192 resulted in neutral or negative outcomes. This finding prompted us to further analyse the
193 correlation between rhizobial colonization and plant growth. We used a subset of 11 *L.*
194 *burttii*-rhizobia combinations, which included strains from the five different genera that
195 formed pink nodules with *L. burttii*. For each of the 11 different *L. burttii*-rhizobia
196 associations, only the shoot length of plants with fully developed pink nodules was compared
197 to mock-treated plants and to plants nodulated by *M. loti* R7A (**Figure 3A**). Additionally, the
198 nodule structure and bacteroid occupancy in the nodule cells were visualized by light
199 microscopy (**Figure 3B-M**). Only *L. burttii* plants harbouring pink nodules colonized by *S.*
200 *americanus* and *A. undicola* (strains #7 and #35) showed comparable shoot growth to plants
201 nodulated by *M. loti* R7A (**Figure 3A**). In contrast, plants nodulated by *Bradyrhizobium* Sp.
202 ORS285 (strain #38) exhibited a significant reduction in the shoot length, while the
203 remaining *L. burttii*-rhizobia interactions did not affect the length of the aerial part (**Figure**
204 **3A**). Nodule sections revealed successful rhizobial colonization by all 11 strains tested,
205 though to different extents. *L. burttii* nodules were heavily colonized by *R. leguminosarum*
206 SM140B, *R. leguminosarum* SM144A and *Bradyrhizobium* ORS285 (**Figure 3G, H and L**),
207 but none of these strains had a positive effect on plant shoot length (**Figure 3A**). These results
208 confirm that the permissive nature of *L. burttii*, which allows successful nodulation by a
209 broad range of rhizobial species, does not always result in growth promotion.

210

211 A single genetic locus near *Nfr1* confers *L. burttii* promiscuity

212 With the exception of *S. fredii* NGR234, Gifu only formed large, pink nodules with its
213 cognate efficient nitrogen fixer *M. loti* that contributed positively to plant growth. The
214 situation was much more complex for *L. burttii*, which engaged in many more different
215 interactions with variable outcomes for the plant. In order to understand the genetics
216 underlying *L. burttii* promiscuity, we inoculated 18 Gifu x *L. burttii* RILs (Sandal et al., 2012)
217 with the 11 diverse strains mentioned above and *R. leguminosarum* Norway, which nodulates
218 *L. burttii* roots intercellularly but does not form nodules with Gifu (Grossmann et al. 2012;
219 Liang et al. 2019) (**Figure 3; Supplementary dataset 1**).

220 We scored the average number of pink nodules for each RIL-rhizobium combination
221 (**Supplemental dataset 1**) and analysed the resulting data using R/qtl. TM0002, the marker
222 previously reported to be associated with the *S. fredii* HH103 Gifu/*L. burttii* QTL (Sandal et
223 al. 2012), was identified as the highest peak in the QTL analyses for all 12 strains (**Figure**
224 **4**). Interestingly, the single locus that appears to be responsible for allowing *L. burttii*
225 nodulation with all 12 strains tested was located on chromosome 1, near the Nod Factor
226 Receptor gene *Nfr1*.

227

228 **A *L. burttii*/Gifu *Nfr1* domain swap construct complements the *nfr1* mutant in a Gifu** 229 **background, but does not extend its host range**

230 Our analysis revealed that an *Nfr1*-linked locus, or *Nfr1* itself, is involved in determining
231 permissiveness towards diverse rhizobial species in *L. burttii*. We sequenced the extracellular
232 region of the *L. burttii Nfr1* gene and found two mis-sense substitutions compared to the Gifu
233 gene (T124A and Y213D, Gifu/*L. burttii*). This made *Nfr1* a strong candidate gene for
234 explaining the capacity of *L. burttii* to establish symbiotic associations with multiple
235 rhizobial genera. To examine this possibility, we created a BinG1 domain swap construct
236 where the *Nfr1* extracellular domain from Gifu was replaced by the corresponding fragment
237 from *L. burttii Nfr1*. This construct was introduced into Gifu wild type and *Ljnfr1-1* mutant
238 backgrounds using *Agrobacterium rhizogenes* hairy root transformation. The *BinG1*
239 construct was functional and rescued Gifu *nfr1-1* nodulation with *M. loti* R7A (**Figure 5**). In
240 contrast, it did not enable either the Gifu wild type or the Gifu *nfr1-1* mutant to nodulate with
241 *S. fredii* HH103 (**Figure 5**). A functional *L. burttii Nfr1* extracellular domain in the Gifu
242 background was thus not sufficient for extending the rhizobial host range of Gifu.

243

244 **Nodulation of *L. burttii* with *R. leguminosarum* Norway is a dominant trait**

245 Recently it was shown that *Rhizobium leguminosarum* Norway colonizes *L. burttii* roots
246 intercellularly (Liang et al. 2019) but is unable to infect *L. japonicus* (Grossman et al. 2012).
247 This report along with the data presented in this study confirms the wide host range of *L.*

248 *burtii* with diverse rhizobial species. To assess if this nodulation promiscuity is a recessive
249 or dominant trait, the F1 progeny of crosses between *L. burtii* and *L. japonicus* Gifu were
250 inoculated with *R. leguminosarum* Norway-GFP. Gifu remained without nodules six weeks
251 post inoculation. The F1 progeny developed nodules regardless of the parental combination
252 (**Figure 6A**) and all phenotyped F1 plants were heterozygous (**Figure 6B**). These results
253 show that the nodulation phenotype of *L. burtii* is dominant.

254

255 **Discussion**

256 The legume-rhizobia symbiosis is an illustrative example of a highly specific and stringent
257 molecular dialogue between symbiotic partners. Despite this fact, it is known that certain
258 rhizobial species such as *S. fredii* NGR234 is able to establish symbiotic associations with
259 diverse legumes from distant phylogenetic genera (Pueppke and Broughton, 1999). In the
260 last decades, both rhizobial and plant components defining the legume-rhizobia compatibility
261 have been identified (Walker et al. 2020). This study shows that a single locus in *L. burtii* is
262 responsible for promiscuous interactions across rhizobial genera.

263

264 ***L. burtii* a promiscuous symbiotic partner engaged in beneficial and detrimental** 265 **associations**

266 *L. burtii* is related to *L. japonicus*, a model legume with extensive genetic and transcriptomic
267 resources (Fukai et al. 2012; Urbański et al. 2012; Mun et al. 2016; Małolepszy et al. 2016;
268 Kamal et al. 2020). Unlike *L. japonicus*, which seems to be nodulated by a narrow range of
269 rhizobial partners, *L. burtii* established 30 associations with diverse rhizobia that culminated
270 in the formation of pink nodules. Excluding the *L. burtii*-*M. loti* interaction, only nine
271 rhizobial species had a marginal but significantly positive contribution to host plant growth
272 while the rest of the rhizobial interactions either did not affect or had a negative impact on *L.*
273 *burtii* growth. Except for *R. leguminosarum* Norway (Liang et al. 2018), the rhizobial strains
274 used in this study are efficient nitrogen fixers in the symbiotic associations with their cognate
275 plant hosts (Cavassim et al. 2020; Moeskjær et al. 2021; Pueppke and Broughton 1999;

276 Dreyfus and Dommergues 1981; Buendia-Claveria et al. 1989; Lajudie et al. 1994; Mora et
277 al. 2014; Lajudie et al. 1998a, 1998b; Gao et al. 2004; Ramírez-Bahena et al. 2009; Gao et
278 al. 2015). The suboptimal outcomes of many of these *L. burttii*-rhizobial associations may
279 be caused by a delay in the nodulation kinetics, as occurs in the *L. burttii*-*S. fredii* HH103
280 symbiosis (Acosta-Jurado et al. 2016b). However, additional compatibility elements, acting
281 at later stages of nodulation could be required for efficient nodule functioning and nitrogen
282 fixation (Walker et al. 2020). Similar examples of inefficient legume-rhizobia symbioses
283 have been documented in *Medicago* spp. *S. meliloti* 1021 is an efficient nitrogen-fixer in
284 *Medicago sativa* nodules and is also able to form pink nodules with the *M. truncatula*
285 accessions A17 and R108, but with poor nitrogen fixation performance (Terpolilli et al. 2008;
286 Kazmierczak et al. 2017). Similarly, ineffective mutants of *S. meliloti* were comparable to
287 their effective counterparts in colonizing *M. sativa* nodules (Amarger 1981). However,
288 legumes possess mechanisms to reward or penalize the effectiveness of their rhizobial
289 partners hosted within nodule cells. In soybean nodules, where inefficient nitrogen fixation
290 was mimicked by substituting nitrogen for argon, the population and growth of rhizobia was
291 drastically lower with respect to nodules where nitrogen fixation was performed efficiently
292 (Kiers et al. 2003). Likewise, four-generation experiments conducted with twelve *M.*
293 *truncatula* genotypes inoculated with a mixture of three rhizobial strains from their native
294 range revealed an increase in the relative frequency of more beneficial rhizobial strains,
295 estimated by the nodule number and size (Heath and Tiffin 2009).

296 A relatively low capacity of legumes to colonize new habitats seems to be related to a scarce
297 presence of compatible symbionts (Parker 2001). This idea is supported by the high
298 invasiveness of certain woody legumes that possess a broad compatibility with diverse
299 rhizobial strains (Richardson et al. 2000). A comparative study among congeneric acacias
300 revealed that those considered invasive associate with a significantly greater number of
301 rhizobial strains than the natural and non-invasive acacias in Australia (Klock et al. 2015).
302 However, studies conducted in distinct geographical regions show that differential
303 invasiveness of *Acacia* species is not always determined by a broad promiscuity with
304 rhizobial strains (Klock et al. 2016; Keet et al. 2017). Similar approaches could be taken with
305 *L. burttii* to further understand the contribution of host range to legume adaptiveness.

306

307

308 **Molecular players restricting the compatibility of legume-rhizobium associations**

309 Root nodule symbiosis encompasses different checkpoints where suitable symbionts are
310 scrutinized, from the early infection to development of functional, nitrogen fixing nodules
311 (Walker et al. 2020). The rhizobial host range is determined by the perception of specific root
312 flavonoids along with certain rhizobial effectors and genes that contribute to NF and
313 exopolysaccharide production (Sugawara et al. 2018; Acosta-Jurado et al. 2016a; Acosta-
314 Jurado et al. 2019, 2020; Kusakabe et al. 2020; Ratu et al. 2021). From the plant side, the
315 first step in symbiotic partner discrimination is the recognition of specific NFs by NF
316 receptors (Radutoiu et al. 2003; Amor et al. 2003; Smit et al. 2007). The next level of
317 selectivity is imposed by scrutiny of exopolysaccharides (EPS) produced by rhizobia. In Lotus,
318 this relies on the EPS receptor *LjEpr3* (Kawaharada et al. 2015) and EPS signalling appears
319 to be of general importance across legumes. The incompatibility of *S. meliloti* Rm41 with *M.*
320 *truncatula* A17 is abolished by incorporating the succinoglycan-coding *exo* gene of the
321 compatible *S. meliloti* 1021 (Simsek et al. 2007). Similarly, EPS composition confers
322 different levels of rhizobial resistance towards the antimicrobial *M. truncatula* nodule-
323 specific cysteine-rich peptides (NCRs) produced in the nodule cells of certain legumes to
324 impose terminal differentiation of bacteroids (Montiel et al. 2017; Arnold et al. 2018). In this
325 regard, the presence of a functional NCR allele in the *M. truncatula* A17 accession restricts
326 its symbiotic association with *S. meliloti* Rm41. This incompatibility is not present in the *M.*
327 *truncatula* DZA315 accession that possesses a non-functional NCR allele (Yang et al. 2017;
328 Wang et al. 2017).

329 The broad host range in *L. burttii* is not explained by any of the plant regulators mentioned
330 above. Unlike legumes of the inverted repeat-lacking clade, where terminal differentiation of
331 bacteroids is orchestrated by NCRs, this peptide family is absent in *Lotus* spp. (Kereszt et al.
332 2018). Our QTL analyses with data generated from 18 Gifu x *L. burttii* RILs inoculated with
333 12 diverse rhizobial strains showed that a locus near microsatellite marker TM0002 confers
334 the symbiotic promiscuity of *L. burttii*. It is unlikely that *LjEpr3* is responsible for the
335 extended nodulation capacity of *L. burttii*, since this gene is not located near TM0002. By
336 contrast, the NF receptor gene *NfrI* was an obvious candidate, since it is located near the
337 TM0002 locus. However, Gifu plants expressing a functional extracellular domain of *L.*

338 *burttii* *Nfr1* did not result in host-range expansion to include nodulation with *S. fredii* HH103.
339 Since *Nfr1* expression was driven by the Gifu *Nfr1* promoter in this experiment and since
340 there may be additional Gifu/*L. burttii* polymorphisms in other *Nfr1* domains, differences in
341 gene sequence or in endogenous *Nfr1* expression levels between *L. burttii* and Gifu may still
342 account for the difference in nodulation phenotype. However, the *L. burttii* symbiotic
343 associations are established with rhizobial strains producing NF with very diverse
344 decorations (**Table 1**) (D’Haeze and Holsters 2002; Bek et al. 2010; Renier et al. 2011),
345 suggesting that minor changes to nod factor receptors may not be the most likely cause of *L.*
346 *burttii* promiscuity. An alternative explanation is that other components linked to the
347 TM0002 marker are responsible, for instance, the presence of several resistance proteins in
348 *G. max* restrict strain specific interactions with rhizobia (Walker et al. 2020). Likewise in
349 *Lotus* accessions it was recently found that *Bradyrhizobium elkanii* USDA61 mutants
350 disrupted in different effector proteins of the type III secretion system are affected at different
351 checkpoints in their symbiotic association with *L. burttii*, *L. japonicus* Gifu and *L. japonicus*
352 MG-20 (Kusakabe et al. 2020). However, the broad host ability of *L. burttii* is unlikely to be
353 linked to a missing R-gene, since the F1 progeny of *L. burttii* and Gifu crosses retained the
354 nodulation capacity with *R. leguminosarum* Norway, while Gifu wile type plants were unable
355 to develop nodules. Therefore, the genetic components responsible for the pronounced
356 symbiotic promiscuity of *L. burttii* remain elusive.

357

358 **Conclusion**

359 In this study we have shown that *L. burttii* exhibits a remarkably broad host range, which is
360 controlled by a single, dominant genetic locus near the TM0002 marker.

361

362 **Author contributions**

363 M.Z., N.S., H.J., Y.-Y.L., E.J. performed experiments. J.S., S.U.A., M.P., M.M. provided
364 resources. M.Z., and J.M. analysed data. S.U.A. supervised the project. M.Z., J.M. and
365 S.U.A. wrote the paper.

366

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369

370 Figure legends

371 **Figure 1. Phylogenetic distribution of rhizobia used in this study.** Phylogenetic tree of
372 Alpha and Beta-rhizobia adapted from Sprent et al. (2017) to highlight the number of species
373 used in this work from each rhizobial genus to evaluate the nodulation capacity of *L.*
374 *japonicus* Gifu and *Lotus burttii*.

375 **Figure 2. Nodulation and shoot phenotypes.** A, Presence (filled rectangles) or absence
376 (unfilled rectangles) of bumps (B), white nodules (W), and pink nodules (P) in Gifu and *L.*
377 *burttii* plants at 5 wpi with 42 different rhizobial strains. In column S, the black and red
378 arrows indicate a significant increase or decrease of the shoot length respect to mock-treated
379 plants, respectively. Student's t test; $P < 0.01$. B-E, Violin dot plots showing the number of
380 bumps, white nodules, pink nodules and shoot length in Gifu and *L. burttii* harvested at 5 wpi
381 with the rhizobial indicated in 2A. Dashed lines in green and orange highlight the average
382 shoot length in mock-treated plants of Gifu and *L. burttii*, respectively.

383 **Figure 3. Shoot length and nodule histology of *L. burttii* plants.** A, Violin dot plots
384 showing the shoot length of *L. burttii* plants with pink nodules at 5 wpi with rhizobial species
385 from different genera. Letters a and c, below the violin plots, indicate non-significant
386 differences between the shoot length compared to mock-treated or R7A-inoculated plants,
387 respectively; b, means significant difference to mock-treated and R7A-inoculated plants.
388 ANOVA, P-Tukey < 0.01 . The colour code reflects the phylogenetic proximity of the species
389 shown in Figure 1. Dashed grey line highlights the average shoot length in mock-treated
390 plants. B-M, Nodule histology with representative images of pink nodules developed in *L.*
391 *burttii* by different rhizobial strains. Scale bar, 1 mm for nodules and 100 μm nodule sections.

392 **Figure 4. QTL analysis of Gifu x *L. burttii* RIL nodulation.** The trait analysed is the
393 average number of pink nodules. A: *S. fredii* HH103. B: *S. fredii* USDA257. C: *S. americanus*
394 CFEI156. D: *R. leguminosarum* SM155B. E: *R. leguminosarum* SM140B. F: *R.*
395 *leguminosarum* SM144A. G: *R. leguminosarum* vici. H: *A. undicola* LMGT. I: *M.*
396 *plurifarum* PMS0804. J: *Bradyrhizobium* sp. ORS285. K: *B. pachyrhizi* PMS0802. L: *R.*
397 *leguminosarum* Norway.

398 **Figure 5. Influence of the *Nfr1* genotype on nodulation with *S. fredii* HH103.** Violin dot
399 plots show the number of pink nodules found in *L. burttii*, Gifu and the *LjNfr1-1* mutant
400 transformed with the *BinG1* and *LjNfr1* constructs or the empty vector at 5 wpi with *M. loti*
401 R7A and *S. fredii* HH103. *BinG1*, *Nfr1* extracellular domain of Gifu replaced by the

402 corresponding fragment from *L. burttii* *Nfr1* expressed under *LjNfr1* promoter. *LjNfr1*, Gifu
403 *Nfr1* sequence expressed under its native promoter. The number of plants tested is indicated
404 below the violin plots.

405 **Figure 6. *L. burttii* x *L. japonicus* Gifu F1 genotype and nodulation phenotype with *R.***
406 ***leguminosarum* Norway.** A, Violin dot plots show the nodule numbers at 6 wpi with *R.*
407 *leguminosarum* Norway in *L. burttii*, *L. japonicus* Gifu and the F1 progeny from crosses
408 between these two genotypes. The number of plants tested are described below the violin dot
409 plots. B, Agarose gel with PCR products amplified with a set of primers for the *Lotus* power
410 marker TM1203 using as template DNA isolated from *L. burttii* (Lb), *L. japonicus* Gifu (Lj
411 Gifu) and the F1 progeny from their crosses (Lb x Lj Gifu).

412 **Supplementary Figure S1. Contrasting nodulation phenotypes in Gifu and *L. burttii***
413 **with different rhizobial species.** Representative images of nodule structures developed in
414 Gifu and *L. burttii* plants at 5 wpi with *S. fredii* NGR234 (A), *S. teranga* LMG7834 (B), *R.*
415 *leguminosarum* SM20 (C), *R. leguminosarum* SM5 (D), *M. plurifarium* PMS0804 (E) and *B.*
416 *pachyrhizi* PMS0802 (F). Scale bar, 1 mm.

417 **Table 1. NF structure in rhizobial strains from different genera.** Composition and
418 chemical decorations in the nonreducing and reducing end of NF produced by rhizobial
419 species used in this study (*) and closely related strains. Table adapted from D’Haeze and
420 Holsters (2002). Abbreviations: Ac, acetyl; Ara, arabinosyl; Cb, carbamoyl; Fuc, fucosyl;
421 Me, methyl; S, sulfate ester; H, hydroxide. The colour code reflects the phylogenetic
422 proximity of the species shown in Figure 1.

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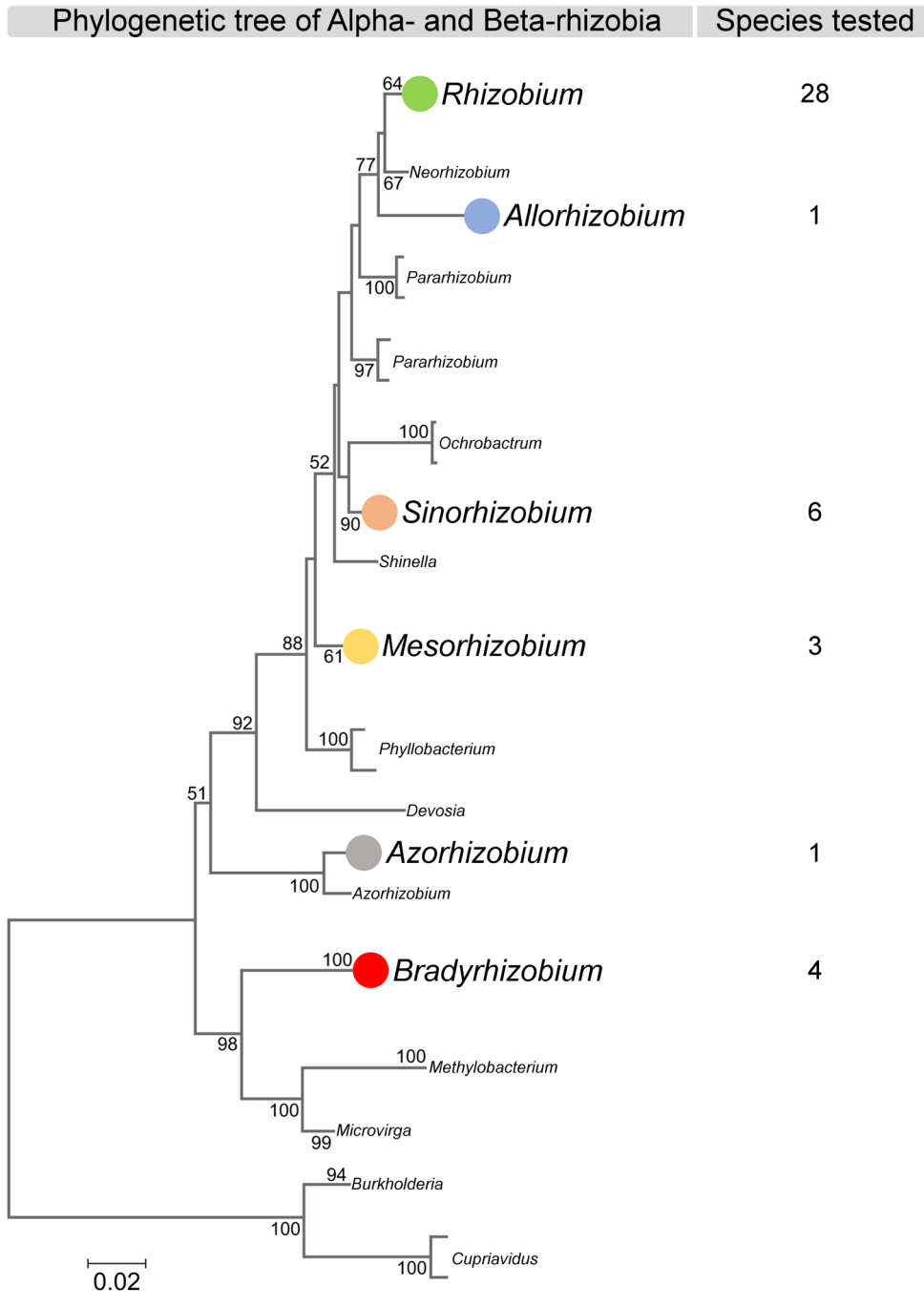
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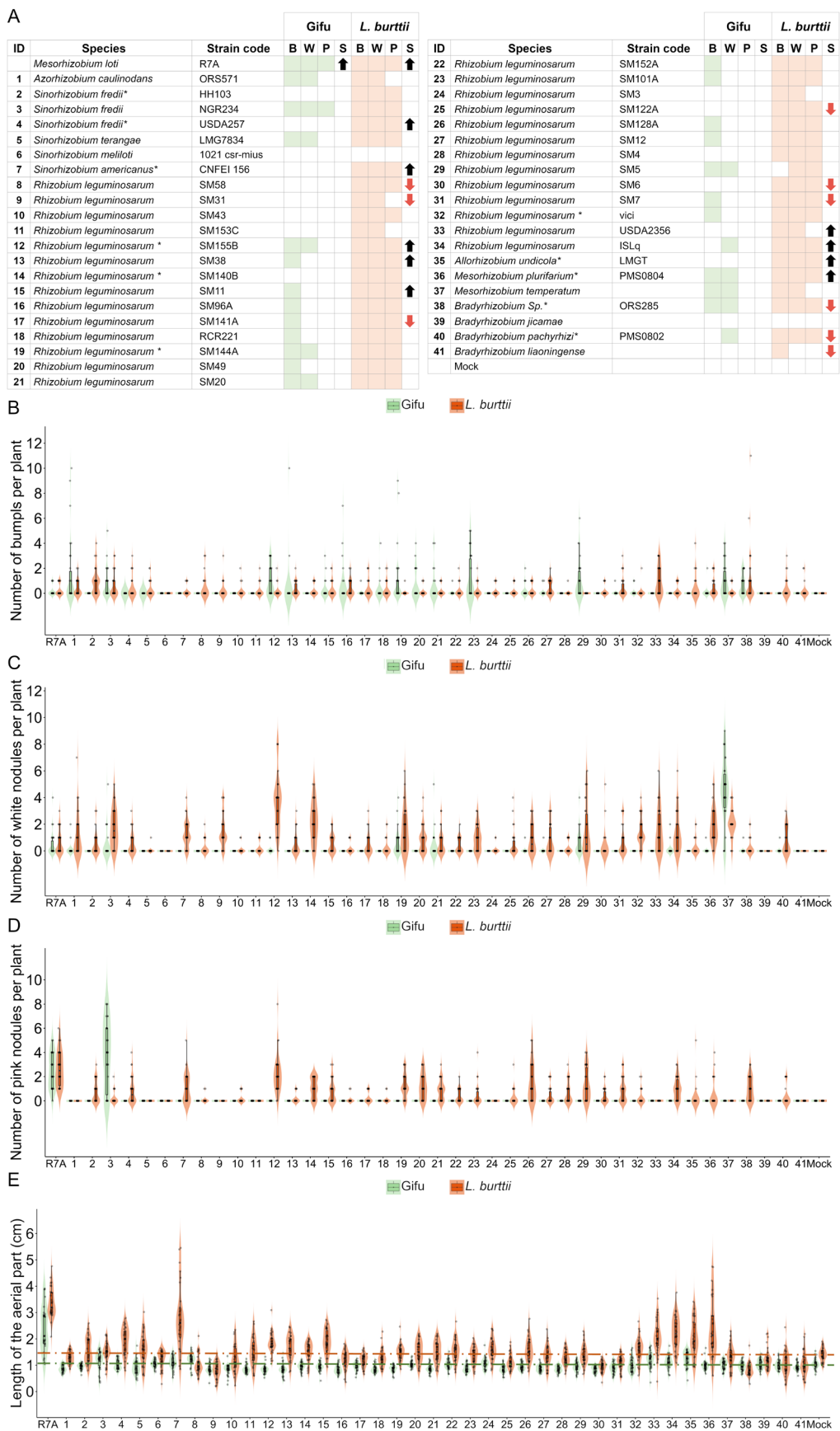
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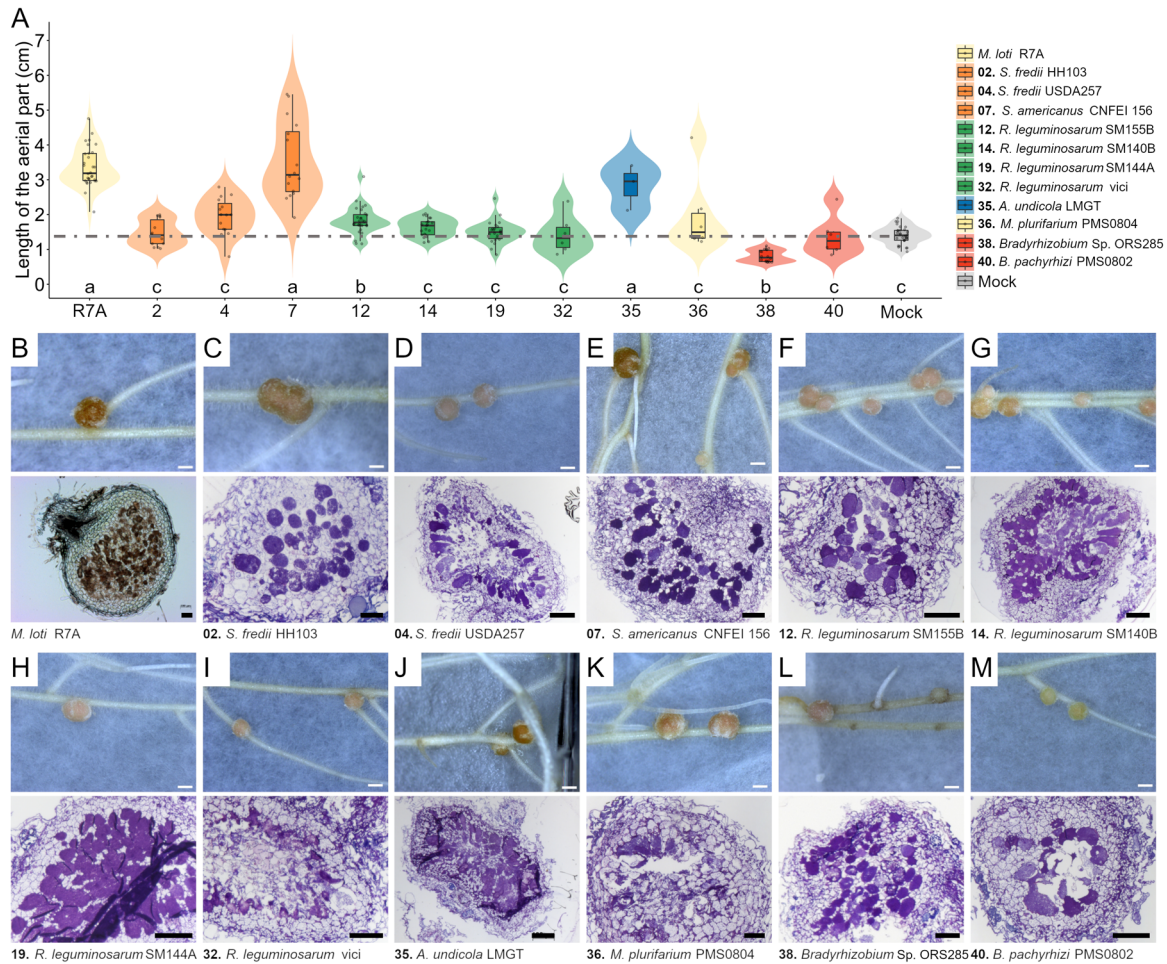


670 **Figure 1. Phylogenetic distribution of rhizobia used in this study.** Phylogenetic tree of
671 Alpha and Beta-rhizobia adapted from Sprent et al. (2017) to highlight the number of species
672 used in this work from each rhizobial genus to evaluate the nodulation capacity of *L.*
673 *japonicus* Gifu and *Lotus burtii*.
674



676 **Figure 2. Nodulation and shoot phenotypes.** **A**, Presence (filled rectangles) or absence
677 (unfilled rectangles) of bumps (B), white nodules (W), and pink nodules (P) in Gifu and *L.*
678 *burttii* plants at 5 wpi with 42 different rhizobial strains. In column S, the black and red
679 arrows indicate a significant increase or decrease of the shoot length respect to mock-treated
680 plants, respectively. Student's t test; $P < 0.01$. **B-E**, Violin dot plots showing the number of
681 bumps, white nodules, pink nodules and shoot length in Gifu and *L. burttii* harvested at 5 wpi
682 with the rhizobial indicated in 2A. Dashed lines in green and orange highlight the average
683 shoot length in mock-treated plants of Gifu and *L. burttii*, respectively.

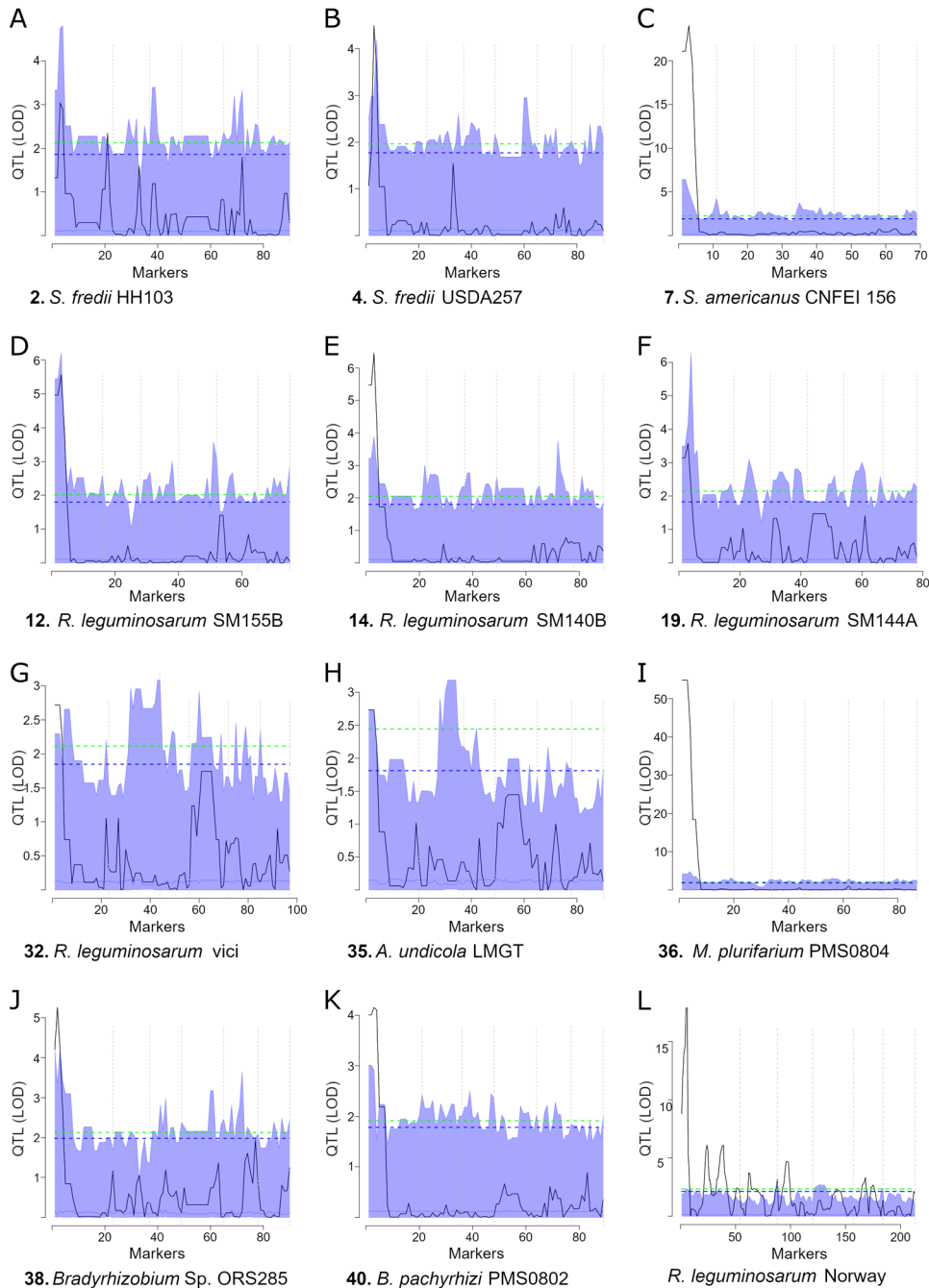
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686 **Figure 3. Shoot length and nodule histology of *L. burttii* plants.** A, Violin dot plots
 687 showing the shoot length of *L. burttii* plants with pink nodules at 5 wpi with rhizobial species
 688 from different genera. Letters a and c, below the violin plots, indicate non-significant
 689 differences between the shoot length compared to mock-treated or R7A-inoculated plants,
 690 respectively; b, means significant difference to mock-treated and R7A-inoculated plants.
 691 ANOVA, P-Tukey < 0.01. The colour code reflects the phylogenetic proximity of the species
 692 shown in Figure 1. Dashed grey line highlights the average shoot length in mock-treated
 693 plants. **B-M**, Nodule histology with representative images of pink nodules developed in *L.*
 694 *burttii* by different rhizobial strains. Scale bar, 1 mm for nodules and 100 µm nodule sections.

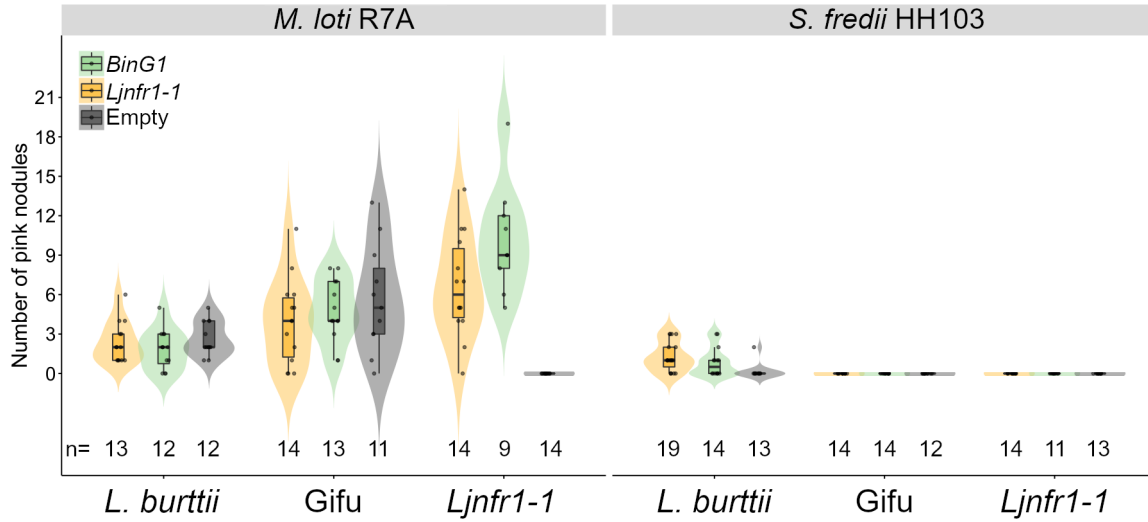
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697 **Figure 4. QTL analysis of Gifu x *L. burttii* RIL nodulation.** The trait analysed is the
 698 average number of pink nodules. A: *S. fredii* HH103. B: *S. fredii* USDA257. C: *S. americanus*
 699 CFEI156. D: *R. leguminosarum* SM155B. E: *R. leguminosarum* SM140B. F: *R.*
 700 *leguminosarum* SM144A. G: *R. leguminosarum* vici. H: *A. undicola* LMGT. I: *M.*
 701 *plurifarium* PMS0804. J: *Bradyrhizobium* sp. ORS285. K: *B. pachyrhizi* PMS0802. L: *R.*
 702 *leguminosarum* Norway.

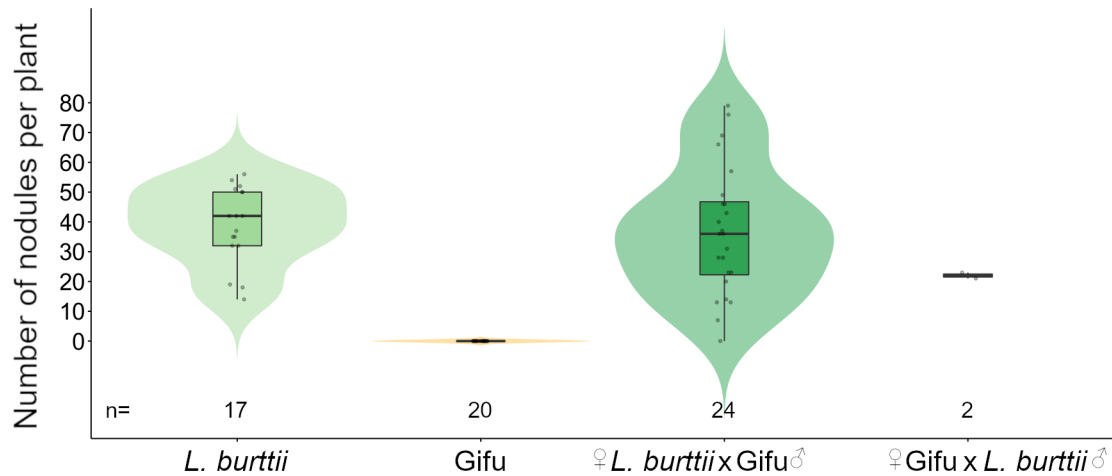
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705 **Figure 5. Influence of the *Nfr1* genotype on nodulation with *S. fredii* HH103.** Violin dot
706 plots show the number of pink nodules found in *L. burttii*, Gifu and the *Ljnfr1-1* mutant
707 transformed with the *BinG1* and *LjNfr1* constructs or the empty vector at 5 wpi with *M. loti*
708 R7A and *S. fredii* HH103. *BinG1*, *Nfr1* extracellular domain of Gifu replaced by the
709 corresponding fragment from *L. burttii* *Nfr1* expressed under *LjNfr1* promoter. *LjNfr1*, Gifu
710 *Nfr1* sequence expressed under its native promoter. The number of plants tested is indicated
711 below the violin plots.

712



713

714 **Figure 6. *L. burttii* x *L. japonicus* Gifu F1 genotype and nodulation phenotype with *R.***
715 ***leguminosarum* Norway.** A, Violin dot plots show the nodule numbers at 6 wpi with *R.*
716 ***leguminosarum* Norway in *L. burttii*, *L. japonicus* Gifu and the F1 progeny from crosses**
717 **between these two genotypes. The number of plants tested are described below the violin dot**
718 **plots. B, Agarose gel with PCR products amplified with a set of primers for the *Lotus* power**
719 **marker TM1203 using as template DNA isolated from *L. burttii* (Lb), *L. japonicus* Gifu (Lj**
720 **Gifu) and the F1 progeny from their crosses (Lb x Lj Gifu).**

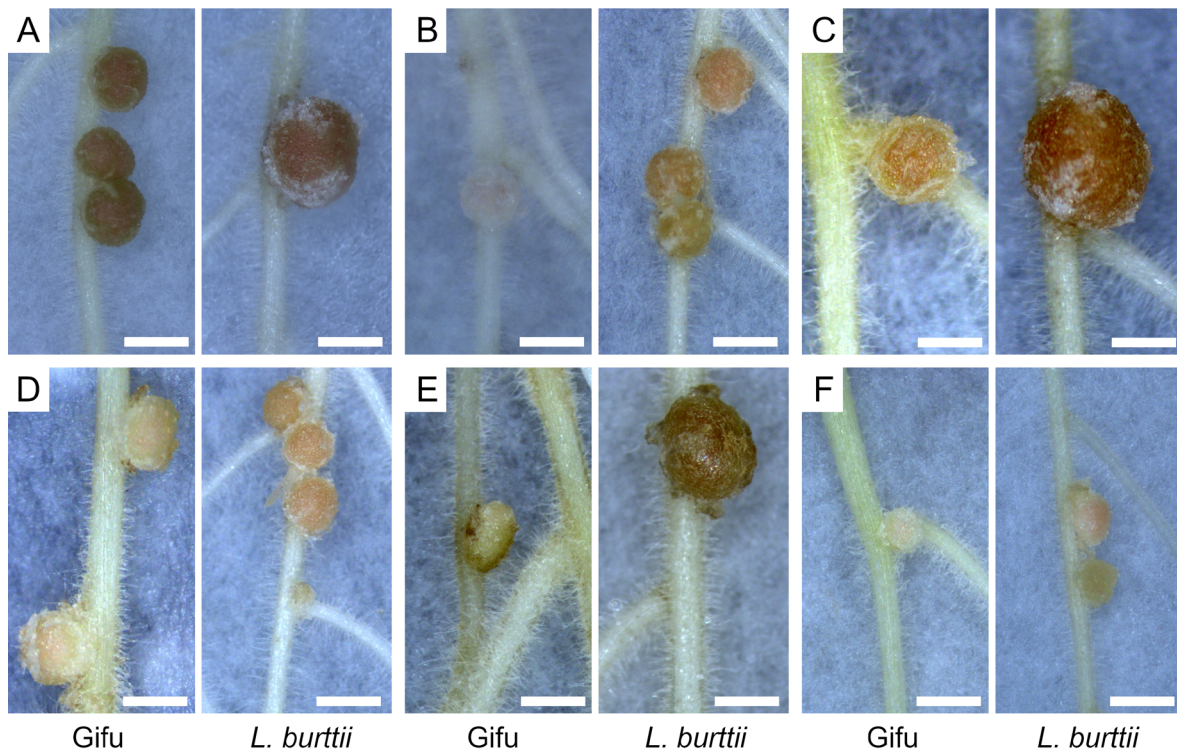
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Rhizobial strain	Nod factor substitutions		
	Acyl group	Non-reducing end	Reducing end
<i>Azorhizobium</i> Sp.			
* <i>A. caulinodans</i> ORS571	C18:1,C18:0	Me, Cb, H	Fuc, Ara, Me, H
<i>Sinorhizobium</i> Sp.			
* <i>Sinorhizobium</i> NGR234	C18:1,C16:0	Me, Cb, H	3-O-S-2-O-MeFuc , Me, H
* <i>S. fredii</i> HH103	C16:0,C16:1	H	2-O-MeFuc, Fuc, Me, H
* <i>S. fredii</i> USDA257	C18:1	H	2-O-MeFuc, Fuc, Me, H
<i>S. teranga</i> <i>bv. acaciae</i> ORS1073	C16:0,C18:0,C18:1	Me, Cb, H	S, H
<i>S. teranga</i> <i>bv. sesbaniae</i>	C16:0,C18:1	Me, Cb, H	Fucy, Ara, Me, H
* <i>S. meliloti</i> RCR2011	C16:1,C16:2,C16:3	Ac, H	Me, H
<i>Rhizobium</i> Sp.			
<i>R. leguminosarum</i> <i>bv. viciae</i> A1	C16:0,C16:1,C18:0	Ac, H	Ac, Me, H
<i>R. leguminosarum</i> <i>bv. viciae</i> RBL5560	C18:4,C18:1,C18:0	Ac, H	Me, H
<i>R. leguminosarum</i> <i>bv. viciae</i> TOM	C18:4,C18:1	Ac, H	Ac, Me, H
<i>Mesorhizobium</i> Sp.			
* <i>M. loti</i> R7A	C16:0,C16:1,C18:0	Me,Cb, Fuc, H	Ac, Fuc, H
<i>Bradyrhizobium</i> Sp.			
<i>Bradyrhizobium</i> strains	C18:1,C18:2	Me, Cb, H	3-O-S-2-O-MeFuc, H
* <i>Bradyrhizobium</i> ORS285	C18:1	Me, Cb, H	2-O-MeFuc, H

723 **Table 1. NF structure in rhizobial strains from different genera.** Composition and
724 chemical decorations in the nonreducing and reducing end of NF produced by rhizobial
725 species used in this study (*) and closely related strains. Table adapted from D’Haeze and
726 Holsters (2002). Abbreviations: Ac, acetyl; Ara, arabinosyl; Cb, carbamoyl; Fuc, fucosyl;
727 Me, methyl; S, sulfate ester; H, hydroxide. The colour code reflects the phylogenetic
728 proximity of the species shown in Figure 1.

729



731 **Supplementary Figure S1. Contrasting nodulation phenotypes in Gifu and *L. burttii***
732 **with different rhizobial species.** Representative images of nodule structures developed in
733 Gifu and *L. burttii* plants at 5 wpi with *S. fredii* NGR234 (A), *S. teranga* LMG7834 (B), *R.*
734 *leguminosarum* SM20 (C), *R. leguminosarum* SM5 (D), *M. plurifarium* PMS0804 (E) and *B.*
735 *pachyrhizi* PMS0802 (F). Scale bar, 1 mm.