Evolution of diverse effective N_2-fixing microsymbionts of *Cicer arietinum* following horizontal transfer of the *Mesorhizobium ciceri* CC1192 symbiosis integrative and conjugative element

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Abstract (250 words)

Rhizobia are soil bacteria capable of forming N_2-fixing symbioses with legumes, with highly effective strains often selected in agriculture as inoculants to maximize symbiotic N_2 fixation. When rhizobia in the genus *Mesorhizobium* have been introduced with exotic legumes into farming systems, horizontal transfer of symbiosis Integrative and Conjugative Elements (ICEs) from the inoculant strain to soil bacteria has resulted in the evolution of ineffective N_2-fixing rhizobia that are competitive for nodulation with the target legume. In Australia, *Cicer arietinum* (chickpea) has been inoculated since the 1970's with *Mesorhizobium ciceri* sv. *ciceri* CC1192, a highly effective strain from Israel. Although the full genome sequence of this organism is available, little is known about the mobility of its symbiosis genes and the diversity of cultivated *C. arietinum*-nodulating organisms. Here, we show the CC1192 genome harbors a 419-kb symbiosis ICE (ICE*Mc*Sym^{1192}) and a 648-kb *repABC*-type plasmid pMC1192 carrying putative *fix* genes. We sequenced the genomes of 11 *C. arietinum* nodule isolates from a field site exclusively inoculated with CC1192 and showed they were diverse unrelated *Mesorhizobium* carrying ICE*Mc*Sym^{1192}, indicating they had acquired the ICE by environmental transfer. No exconjugants harboured pMc1192 and the plasmid was not essential for N_2 fixation in CC1192. Laboratory conjugation experiments confirmed ICE*Mc*Sym^{1192} is mobile, integrating site-specifically within the 3' end of one of the four *ser*-tRNA genes in the R7ANS recipient genome. Strikingly, all ICE*Mc*Sym^{1192} exconjugants were as efficient at fixing N_2 with *C. arietinum* as CC1192, demonstrating ICE transfer does not necessarily yield ineffective microsymbionts as previously observed.

Importance (146 words)

Symbiotic N_2 fixation is a key component of sustainable agriculture and in many parts of the world legumes are inoculated with highly efficient strains of rhizobia to maximise fixed N_2 inputs into farming systems. Symbiosis genes for *Mesorhizobium* spp. are often encoded chromosomally within mobile gene clusters called Integrative and Conjugative Elements or ICEs. In Australia, where all

agricultural legumes and their rhizobia are exotic, horizontal transfer of ICEs from inoculant *Mesorhizobium* strains to native rhizobia has led to the evolution of inefficient strains that outcompete the original inoculant, with the potential to render it ineffective. However, the commercial inoculant strain for *Cicer arietinum* (chickpea), *M. ciceri* CC1192, has a mobile symbiosis ICE (ICE*Mc*Sym^{1192}) which can support high rates of N_2 fixation following either environmental or laboratory transfer into diverse *Mesorhizobium* backgrounds, demonstrating ICE transfer does not necessarily yield ineffective microsymbionts as previously observed.

Introduction

Rhizobia are soil bacteria capable of forming a symbiotic association with legumes. The symbiosis is established when rhizobia infect legume roots, resulting in the formation of root nodules, where atmospheric N_2 is "fixed" by rhizobia into organic nitrogen that is incorporated into plant tissues. When grown in rotation with other crops, legume nitrogen enters soil following plant senescence and decay or deposition from grazing livestock (1). Legumes and rhizobia are often introduced into agriculture to increase soil fertility and reduce use of industrially-synthesised nitrogenous fertilisers (2, 3). In Australia, all forage and grain legumes are exotic, having been introduced following European colonization in the late 18^{th} century (4, 5). Prior to this, it appears Australian soils lacked rhizobia capable of forming effective N_2-fixing symbioses with introduced legumes (6, 7). Therefore, many inoculant strains have been sourced from other parts of the world through dedicated selection programs to match plant host with rhizobia that are well-adapted to these environments and capable of fixing high amounts of N_2 with the target legume (7-9).

Since the 1990's, it has become increasingly clear that the genetic diversity of strains resident in Australian soils capable of nodulating agricultural legumes far exceeds the diversity of strains introduced as inoculants (10-13). For some legumes, these resident or "naturalized" rhizobia, are present in such high numbers that they, and not the inoculant strain, dominate as nodule occupants (14, 15). These resident strains often fix N_2 suboptimally (16-18), posing a significant constraint to maximizing symbiotic N_2 fixation. While the origin of resident strains has been debated for some time (Howieson and Ballard, 2004), mounting evidence for the role of horizontal gene transfer as a driver for bacterial evolution (19-21) indicates this is likely a key contributor to their evolution.

Rhizobial symbiosis genes, which include the nodulation (*nod, noe* and *nol* or collectively *nod*) (22), and nitrogen fixation (*nif* and *fix*) genes, are critical to the establishment of N_2-fixing legume associations (23). Symbiosis genes can be carried on plasmids or clustered on the bacterial chromosome, and this arrangement appears to be largely genus specific (24, 25). For rhizobia in the

genus *Mesorhizobium*, symbiosis genes are encoded chromosomally within Integrative and Conjugative Elements or ICEs which can be either monopartite or tripartite in structure (26-28).

The first symbiosis ICE discovered was the monopartite ICE*Ml*Sym^{R7A} in *M. japonicum* (formerly *M. loti*) R7A (27, 29), following inoculation of the introduced legume *Lotus corniculatus* in New Zealand. Rhizobia isolated several years later from *L. corniculatus* root nodules were genetically distinct to the inoculant strain yet harboured the R7A symbiosis ICE (30). ICE*Ml*Sym^{R7A} is integrated at the 3'-end of the *phe*-tRNA gene in the R7A chromosome, flanked by attachment sites (*attL* and *attR*) that contain an identical 17-bp sequence (26) . Excision of ICE*Ml*Sym^{R7A} from the chromosome is catalysed by integrase (IntS) and recombination directionality factor (RdfS)-mediated site-specific recombination between *attL* and *attR*. The resulting episome can subsequently transfer by conjugation to a recipient cell, integrating by IntS-dependent recombination at the 3' end of the *phe*-tRNA gene (26). Importantly, field experiments at a second site in New Zealand showed that over a four-year period, 75% of root nodules sampled contained diverse *Mesorhizobium* strains that had acquired ICE*Ml*Sym^{R7A}, converting them into *L. corniculatus*-nodulating microsymbionts and out-competing the inoculant for nodulation of the legume (31).

Novel microsymbionts have also evolved in Australian soils after introduction of the pasture legume *Biserrula pelecinus* and *M. ciceri* sv. biserrulae WSM1271 from the Mediterranean to western Australia (18, 32, 33). Six years after inoculation at a site with no pre-existing *B. pelecinus* nodulating rhizobia, novel isolates genetically distinct to the inoculant were recovered from *B. pelecinus* nodules. Crucially, all the novel isolates were either completely ineffective (i.e. nodulated *B. pelecinus* but did not fix N_2) or fixed significantly less than WSM1271 (18). Detailed analysis of two strains (*M. australicum* WSM2073 and *M. opportunistum* WSM2075) showed they both had acquired the WSM1271 tripartite symbiosis ICE (ICE*Mc*Sym^{1271}) by horizontal transfer (28). A similar analysis following inoculation of *B. pelecinus* with *M. ciceri* sv. biserrulae WSM1497 also found that 47.5% of strains isolated from nodules were novel and all of these were less effective than WSM1497 at fixing

111 N_2, with six being completely ineffective (18). Therefore, horizontal transfer of symbiosis ICEs from
112 *Mesorhizobium* inoculants has resulted in the evolution of novel strains that are competitive, but
113 less effective at fixing N_2 than the inoculant strain.

114 *Cicer arietinum* (chickpea) is the largest legume crop in Australia (34), predominantly grown in north-
115 eastern Australia, extending to regions in the south-east as well as parts of western Australia. *C.*
116 *arietinum* forms a N_2-fixing symbiosis with rhizobia in the genus *Mesorhizobium* (35), however, when
117 this grain legume was first introduced in the 1970's, Australian soils appeared to lack compatible *C.*
118 *arietinum*-nodulating rhizobia (Corbin, et al. 1977). This led to the selection of strain *M. ciceri* sv.
119 ciceri CC1192 from Israel and its subsequent use as a commercial inoculant for *C. arietinum* across
120 the country for more than 40 years (7, 15, 36). Despite this, Elias and Herridge (37) reported that
121 53% of *C. arietinum* nodules sampled from 26 farms in eastern Australia were occupied by strains
122 genetically distinct to CC1192. Furthermore, 41% of a subset of these strains were significantly less
123 effective at fixing N_2 with *C. arietinum* than CC1192 (37).

124 The presence of soil populations of suboptimally effective *C. arietinum*-nodulating rhizobia could reduce
125 the benefits to agriculture of symbiotic N_2 fixation following inoculation with CC1192. Here, we
126 investigate the genome of CC1192 and describe the structure and genetic content of its symbiosis
127 ICE, ICE*Mc*Sym^{1192}. We show that genes essential for symbiosis are encoded on ICE*Mc*Sym^{1192} and
128 that it is transferable *in vitro* and in the field. Remarkably, transfer of the ICE into genetically diverse
129 *Mesorhizobium* yields strains that are effective microsymbionts of *C. arietinum*, showing that ICE
130 transfer does not always yield inefficient N_2-fixing strains.

131 **Results**

132 **Genetically diverse strains of *Mesorhizobium* nodulate *C. arietinum***

133 To investigate the genetic diversity of rhizobia isolated from field-cultivated *C. arietinum*, we whole
134 genome sequenced 11 strains (WSM4303-WSM4308, WSM4310-WSM4313 and WSM4315) available

135 from the work of Elias and Herridge (37). We compared these sequences to selected *Mesorhizobium*
136 type strains, commercial inoculants released in Australia and strains from the recent study of *Cicer*-
137 nodulating rhizobia by Greenlon *et al*. (38), by constructing a genome tree with bcgTree
138 (Ankenbrand and Keller, 2016) based on 107 essential single-copy core genes (Figure 1). The 11 *C.*
139 *arietinum* strains grouped into three clades, which were not closely related to the commercial
140 inoculant strains for *C. arietinum* (*M. ciceri* sv. ciceri CC1192), *Biserrula pelecinus* (*M. ciceri* sv.
141 biserrulae WSM1497 and WSM1271) or *Lotus* spp. (*M. loti* SU343). The largest clade contained eight
142 of the strains subdivided into five subgroups (WSM4307 with WSM4315, WSM4304 with WSM4308,
143 WSM4305 with WSM4311, and with WSM4310 and WSM4312 each on a separate branch), while
144 WSM4306 and WSM4303 grouped together on a separate branch, only distantly related to other
145 strains in the tree. WSM4313 grouped separately and was most closely related to several strains
146 isolated from *C. arietinum* growing in Ethiopia (*Mesorhizobium* sp. M2D, M2E and M2A), Turkey
147 (*Mesorhizobium* sp. M2C) and *M. plurfarium* from *Acacia senegal* in west Africa.

148 Whole genome sequences are not available for *Mesorhizobium* isolated from Australian native
149 legumes, so we were unable to extend this core-gene analysis to these organisms. Instead, we
150 constructed a 16S rRNA tree using the 11 strains, available sequences from native legume-
151 nodulating *Mesorhizobium* strains, along with commercial inoculant and selected types strains from
152 the genome tree (Figure 2). Consistent with the genome tree, the 11 strains grouped into the same
153 three clades, with the largest clade clustered with *Mesorhizobium* strains U and T, isolated from the
154 native legumes *Acacia obliquinervia* and *Goodia lotifolia* from south-eastern Australia (39). Similarly,
155 WSM4313 grouped closely with *Mesorhizobium* grouping T19, representing a genospecies
156 comprising 39 strains isolated from native legumes *Acacia stenophylla* and *A. salicium* growing in the
157 Murray River Basin area of south-eastern Australia (40), while the remaining two strains (WSM4303
158 and WSM4306) did not group closely with the *Mesorhizobium* strains from Australian native
159 legumes. The 11 strains therefore appeared to be novel *C. arietinum*-nodulating *Mesorhizobium*.
160 Given that Australian soils were reported to lack compatible *C. arietinum*-nodulating rhizobia prior to

161 the release of CC1192 (Corbin, et al. 1977), this suggested these 11 strains may have acquired their
162 symbiosis genes following introduction of the inoculant strain.

163

164 **Environmental and laboratory transfer of ICE*Mc*Sym^{1192} produces effective symbionts**

165 Previous work had predicted CC1192 to harbour a 419-kb symbiosis ICE within its 6.29 Mbp
166 chromosome (Genbank accession number: CP015062) (Haskett et al., 2016b), herein referred to as
167 ICE*Mc*Sym^{1192}. ICE*Mc*Sym^{1192} is a monopartite ICE flanked by 20-bp repeat DNA sequences (5'-
168 GAATCCCTCCCTCTCCGCCA-3'), identical to the 3' end of the *ser*-tRNA gene, which presumably
169 contains the core regions of the integrase attachment sites *attL* and *attR* (Figure 3). The genetic
170 complement of ICE*Mc*Sym^{1192} is broadly similar to that of the well-characterised ICE*Ml*Sym^{R7A} from
171 *M. japonicum* R7A. ICE*Mc*Sym^{1192} harbours a complete set of core nodulation (*nod*) genes and
172 nitrogen fixation (*nif* and *fix*) genes as well as an integrase gene (*intS,* see Table S1 for gene
173 coordinates) distinct to previously characterised symbiosis ICEs, and conjugation (*trb*-gene cluster
174 and *rlxS*) and ICE excision regulation genes (*rdfS*, *fseA*) (Figure 3) highly similar to those of
175 ICE*Ml*Sym^{R7A} and the tripartite ICE*Mc*Sym^{1271}. These characteristics suggested that ICE*Mc*Sym^{1192} is
176 mobile, so we compared the *de novo* assembled genomes of the novel *Mesorhizobium* strains with
177 the CC1192 genome. The entire 419-kb ICE*Mc*Sym^{1192} region was present in all 11 strains, confirming
178 the environmental transfer of ICE*Mc*Sym^{1192} to these strains (Figure 4). We next investigated how the
179 *Mesorhizobium* recipients of ICE*Mc*Sym^{1192} performed in controlled glasshouse experiments on *C.*
180 *arietinum*. Of the 11 strains tested against CC1192, all strains produced foliage dry weights that were
181 not significantly different to CC1192, with mean values ranging from 0.402 to 0.506 g/plant (Figure
182 5). Therefore, these data demonstrate these novel *Mesorhizobium* strains have acquired
183 ICE*Mc*Sym^{1192} and are fully effective at fixing N_2 on *C. arietinum*.

184 We next tested the mobility of ICE*Mc*Sym^{1192} *in vitro*, by carrying out conjugation experiments using
185 R7ANS (the ICE-cured derivative of *M. japonicum* R7A) as a recipient (26). The neomycin-resistance
186 plasmid pPR3 was introduced into R7ANS to enable selection against donor cells. While R7ANS is
187 auxotrophic for biotin, nicotinate and thiamine, the genes for the biosynthesis of these vitamins
188 (*bioBFDAZ*, *nadABC* and *thiCOSGED*, respectively) are present on ICE*Mc*Sym^{1192} (Figure 3). R7ANS
189 exconjugants harbouring ICE*Mc*Sym^{1192} were isolated on medium lacking biotin and nicotinate.
190 Vitamin prototrophs were acquired on selection plates, with a transfer frequency of 1.02 ± 0.52
191 (standard error of the mean) x 10^{-7} exconjugants per donor.

192 As with the CC1192 genome where there are multiple *ser*-tRNA genes (41), five *ser*-tRNA genes are
193 encoded in the R7ANS genome, which are potential integration sites for ICE*Mc*Sym^{1192}, although only
194 one (R7A2020_05665) contains a 20-bp region identical to those present in the *attL* and *attR* sites
195 flanking ICE*Mc*Sym^{1192}. This indicated that this *ser*-tRNA was a likely integration site for ICE*Mc*Sym^{1192}
196 in the R7ANS genome. PCR screening of ten exconjugants with primers designed to target
197 integration of ICE*Mc*Sym^{1192} at R7A2020_05665 showed all ten had integrated at this site. To confirm
198 that mating of CC1192 with R7ANS had resulted in the complete transfer of ICE*Mc*Sym^{1192} into the
199 recipient strain, two exconjugants strains, MCC110 (JADAMJ000000000) and MCC111
200 (JADAMK000000000), were selected for whole genome sequencing. BLASTN comparison of the *de*
201 *novo* assembled genomes with wild-type CC1192 confirmed that the 419-kb ICE*Mc*Sym^{1192} had been
202 transferred in its entirety into the R7ANS recipient, integrating at *ser*-tRNA (R7A2020_05665) (Figure
203 4). Therefore, ICE*Mc*Sym^{1192} is a mobile element able to be acquired by the R7ANS recipient strain.

204 In previous work with the *B. pelecinus*-nodulating strain *M. ciceri* sv. biserrulae WSM1271,
205 laboratory transfer of the WSM1271 tripartite symbiosis ICE*Mc*Sym^{1271} to R7ANS produced
206 exconjugants that were only partially effective at fixing N_2 with the legume host (41). To see if the
207 R7ANS exconjugants that had acquired ICE*Mc*Sym^{1192} similarly show reduced effectiveness, we
208 examined the symbiotic phenotype of the R7ANS exconjugants MCC110 and MCC111 in comparison

209 with CC1192 on *C. arietinum*, along with R7ANS and its parent strain *M. japonicum* R7A. Both R7A
210 and R7ANS failed to nodulate *C. arietinum* and plant shoot dry weights were not significantly
211 different to uninoculated N-starved controls (Figure 6a). In contrast, *C. arietinum* inoculated with
212 MCC110 or MCC111 produced plants with more than 3.5-fold greater shoot biomass than the N-
213 starved control. These values were not significantly different ($P \leq 0.05$) to the mean shoot dry weights
214 of CC1192-inoculated plants. Although MCC110 and MCC111 produced approximately 23% more
215 nodules ($P < 0.05$) than CC1192 on *C. arietinum*, total nodule mass per plant was not different ($P =$
216 0.115) (Figure 6b). Similarly, comparison of nitrogenase activity between wild-type and exconjugant
217 strains by acetylene reduction assays showed that acetylene reduction rates per plant, per nodule
218 and per unit nodule mass were not significantly different across the three strains (Table 1).
219 Therefore, the transfer of ICE*Mc*Sym^{1192} from CC1192 into R7ANS yields exconjugants that form a
220 symbiosis that is equally effective at fixing N_2 with *C. arietinum* as wild-type CC1192.

221

222 **The 648-bp plasmid pMc1192 is not essential for N_2 fixation with *C. arietinum***

223 Although acquisition of the symbiosis genes encoded on ICE*Mc*Sym^{1192} is sufficient to support
224 CC1192-levels of N_2 fixation in the 11 novel strains of *Mesorhizobium* and R7ANS(ICE*Mc*Sym^{1192})
225 exconjugants, further sequence analysis of the CC1192 complete genome showed additional
226 putative symbiosis-related genes encoded on the *repABC*-type plasmid, pMc1192 (Accession number
227 CP015063). Among the 645 predicted coding sequences on this 648,231-bp plasmid are copies of
228 *fixNOQP* (A4R28_RS30260, RS30265, RS30270, RS30275) and *fixGHI* (A4R28_RS30280, RS30285,
229 RS30290). These copies are in addition to those on located on ICE*Mc*Sym^{1192} (*fixNOQP*,
230 A4R28_RS20715, RS20710, RS20705, RS20700 and *fixGHI*, A4R28_RS20695, RS20690, RS20685), with
231 which they share an average pairwise nucleotide identity of 88%. The plasmid-encoded *fixGHI* gene
232 cluster also includes *fixS* (A4R28_RS30295), a gene which is absent from the ICE-encoded copy of
233 *fixGHI* (Figure 3). Furthermore, pMc1192 also harbours *fixLJ* (A4R28_30350, A4R28_30355) and *fixK*

(A4R28_30370), which are not present on ICE*Mc*Sym[1192]. *fixLJ* and *fixK* are essential for N_2 fixation in the *S. meliloti-Medicago* symbiosis, with FixLJ acting as a low O_2-sensing two component sensor-regulator system, which in turn controls expression of transcriptional regulators *fixK* and *nifA* (42).

To determine whether the plasmid-encoded *fix* genes had a role in the CC1192-*C. arietinum* symbiosis, plasmid pMc1192 was cured from CC1192 using a plasmid incompatibility approach. Two independently acquired plasmid cured derivatives of CC1192 (MCC69 and MCC70) were isolated. PCR screening, Eckhardt gel electrophoresis (Figure S1) and whole genome sequencing of MCC70 (accession number: SRX9131521) confirmed loss of pMc1192. To determine whether the loss of pMc1192 and the symbiotic genes it contained affected symbiotic performance, the nodulation and N_2 fixation phenotype of the two plasmid-cured strains, MCC69 and MCC70, was compared to that of the parent strain CC1192 on *C. arietinum* (Figure 7). At 44 days post-inoculation, there was no significant difference ($P \leq 0.05$) between MCC69, MCC70 and CC1192 in either mean shoot dry weight or mean nodule dry weight per plant. Furthermore, pMc1192 was also absent from the genomes of the 11 *Mesorhizobium* strains isolated from field-cultivated *C. arietinum*, indicating they had not acquired this plasmid. Therefore, although plasmid pMc1192 harbours predicted *fix* genes, they are not essential to support N_2 fixation on *C. arietinum*.

Discussion

We have demonstrated that *Mesorhizobium ciceri* sv. ciceri CC1192 harbours a 419-kb monopartite symbiosis ICE*Mc*Sym[1192] integrated at one of the four *ser*-tRNA genes in the CC1192 genome. The ICE is transferable to the ICE-devoid R7ANS recipient, where it integrates into a 20-bp sequence present at the 3' end of the *ser*-tRNA gene. Furthermore, the identification of ICE*Mc*Sym[1192] in the genomes of genetically distinct *Mesorhizobium* present in *C. arietinum* nodules is consistent with ICE*Mc*Sym[1192] transfer in the field. Although *ser*-tRNA had been proposed as the integration site for ICE*Mc*Sym[1192] (41) as well as for ten other predicted monopartite symbiosis ICEs from *Cicer*-nodulating strains (38), here we have experimentally confirmed this locus to be an integration site

for symbiosis ICEs. The rate of transfer of ICE*Mc*Sym^{1192} to R7A of 1.05 x 10^{-7} transconjugants per donor is comparable to the ICE*Ml*Sym^{R7A} transfer rate in wild-type *M. japonicum* R7A (2.5 x 10^{-7} per donor) (43), but is more than an order of magnitude higher than for the transfer of ICE*Mc*Sym^{1271} from WSM1271 to R7ANS (4.65 x 10^{-8} per donor) (28). The differences in these transfer rates may be related to monopartite structure of ICE*Mc*Sym^{1192} and ICE*Ml*Sym^{R7A} and their highly similar complement of ICE regulatory genes, compared to the more complex and intricate control system in the tripartite ICE*Mc*Sym^{1271} (44).

Curing CC1192 of plasmid pMc1192 had no impact on the nodulation or N_2 fixation phenotype of the resultant strains and the genomes of the 11 *Mesorhizobium* strains isolated from field-cultivated *C. arietinum* lacked the plasmid. This indicates that the 645 genes, including *fix* genes (*fixNOPQGHIS* and *fixLJK*), encoded on pMc1192 are not essential for symbiosis with *C. arietinum*. In several rhizobia, FixNOQP and FixGHI are required for production of a high-O_2-affinity cbb_3-type cytochrome oxidase critical for symbiosis, with mutations in these operons either abolishing or greatly reducing rates of N_2 fixation (45-48). Given that *fixNOQP* and *fixGHI* are also present on ICE*Mc*Sym^{1192}, it is likely that *M. ciceri* CC1192 relies on these ICE-encoded genes to fulfil the symbiotic roles of FixNOQP and FixGHI. Although a definitive role for FixS is yet to be reported for N_2-fixing rhizobia, *fixS/ccoS* has been shown to be essential for maturation of the microaerobic terminal oxidase cbb_3 complex in the photosynthetic purple non-sulfur bacterium *Rhodobacter sphaeroides* (49, 50). Plasmid pMc1192 appears to harbour the sole copy of *fixS* in the CC1192 genome, therefore either FixS is dispensable for N_2 fixation in this symbiosis or an unidentified gene in CC1192 complements its function. Other *M. ciceri* strains have a similar-sized *repABC*-type plasmid in their genomes, including *M. ciceri* WSM1271, WSM1284 and WSM1497 (41, 51, 52), so it is possible that these replicons may be widely conserved among this species.

The N_2 fixation efficiency of both R7ANS and field-isolated exconjugants harbouring ICE*Mc*Sym^{1192}-was indistinguishable from that of CC1192 on *C. arietinum*. Importantly, ICE*Mc*Sym^{1192} was identified

in all field-isolates tested and these 11 strains were distributed across three different clades, based on 16S rRNA and core gene phylogenies, indicating that this ICE can support CC1192-rates of N_2 fixation in a comparatively wide range of *Mesorhizobium* genetic backgrounds. This is in stark contrast to results obtained previously with WSM1271 and WSM1497 where field-isolated (18, 53) and *in vitro* R7ANS exconjugants of WSM1271 (28) were less effective than the inoculant strains at fixing N_2 with *B. pelecinus*, with some field-isolated strains being completely ineffective (18). Therefore, acquisition of a symbiosis ICE from a *Mesorhizobium* strain can yield novel microsymbionts which are as effective as the inoculant strain at fixing N_2 with host legumes.

Although the 11 *Mesorhizobium* strains isolated from field-cultivated *C. arietinum* showed symbiotic effectiveness equivalent to the inoculant strain CC1192, there is evidence that suboptimally effective strains nodulate *C. arietinum* in Australia. In their study of cultivated-*C. arietinum* inoculated with CC1192, Elias and Herridge (37) isolated 570 strains from nodules of *C. arietinum* sampled across 26 farms in eastern Australia and found that 86% of strains were not CC1192. Importantly, while most novel strains analysed were as effective as CC1192 on *C. arietinum*, 41% were suboptimal at fixing N_2. Although the symbiosis ICE was not identified in these strains, the fact that uninoculated *C. arietinum* sown into fields in Australia without a history of inoculation fails to form effective N_2-fixing nodules (7), is strong evidence for a lack of compatible pre-existing soil organisms capable of fixing N_2 with this legume. Therefore, it is highly likely that the novel *C. arietinum*-nodulating organisms reported by Elias and Herridge (37) are the result of transfer of the CC1192 ICE into recipient soil *Mesorhizobium* spp., as was shown to be the case with the 11 strains analysed in this work.

The field-isolated recipients of ICE*Mc*Sym^{1192} were shown to form three separate clades, some of which may constitute new species of *Mesorhizobium*. Very little is known of the diversity of *Mesorhizobium* soil populations in Australia, with *Bradyrhizobium* spp. most frequently being identified from isolations made from nodules of native legumes (54-57). Only two studies report the isolation of limited numbers of *Mesorhizobium* strains from some species of Australian native

309 legumes (39, 40), and the 16s rRNA sequences of these strains group closely to some of the *C.*
310 *arietinum* field isolated strains analysed in this study. It is possible that the novel *C. arietinum* strains
311 analysed in this study may have evolved from the transfer of ICE*Mc*Sym^{1192} from CC1192 into
312 *Mesorhizobium* microsymbionts of Australian native legumes, converting them into *C. arietinum*
313 symbionts. However, there is no evidence for non- ICE*Mc*Sym^{1192}-encoded symbiosis genes in the
314 genomes of the field isolates, as might be expected if the ICE had integrated into the genome of a
315 pre-existing symbiont. This therefore suggests that the recipients of ICE*Mc*Sym^{1192} may instead have
316 been non-symbiotic *Mesorhizobium* saprophytes existing as part of the soil microbiota. In fact, non-
317 symbiotic *Mesorhizobium* spp. have been isolated from the rhizosphere of *L. corniculatus* growing at
318 two separate field sites in New Zealand (58). It is therefore possible that populations of non-
319 symbiotic *Mesorhizobium* similarly exist in Australian soils and that they can act as recipients for
320 symbiosis ICE transfer from *Mesorhizobium* inoculant strains.

321 Why ICE transfer into different *Mesorhizobium* strains sometimes leads to ineffective or poorly
322 effective N_2-fixing microsymbionts, such as for *B. pelecinus* (52, 53, 59, 60), or effective N_2-fixing
323 rhizobia as presented in this study, is not clear. However, the interaction between expression of ICE
324 and chromosomally encoded genes required for symbiosis is likely to play a significant role. The
325 reports of large "naturalized" populations of *Sinorhizobium* and *Rhizobium* strains present in
326 Australian soils (12, 15, 17) may similarly be the result of horizontal transfer of symbiotic plasmids
327 from inoculant strains to native soil bacteria. Determining how these populations of rhizobia have
328 developed is crucial to continuing to harness rhizobia-legume interactions in farming systems to
329 maximise nitrogen inputs and in understanding the selective forces driving evolution of symbiotic N_2
330 fixation.

331

Experimental Procedures

Strains and plasmids and media

Bacterial strains and plasmids used in this study are detailed in Table 2. *Mesorhizobium* spp. were cultured at 28°C on ½ Lupin agar (LA) (61, 62) or tryptone yeast extract (TY) (63). For ICE transfer experiments, exconjugants were selected on rhizobium defined medium (RDM) supplemented with 15 mM glucose (64). *Escherichia coli* was cultured at 37°C on Lysogeny Broth (LB) medium (65). Where appropriate, antibiotics were supplied in the medium at the following concentrations (in µg ml^{-1}) for *Mesorhizobium* (Neomycin, 250 for exconjugant selection, and 80 for routine culturing; gentamycin 40; tetracycline 1) and *E. coli* (kanamycin, 40; gentamycin 10; tetracycline 10). Media for *E. coli* ST18 was also supplemented with 50 µg ml^{-1} of 5-aminolevulinic acid.

Curing pMc1192 from CC1192

Plasmid pMc1192 was cured from *M. ciceri* CC1192 using a plasmid incompatibility approach by cloning the *repABC* region from pMESCI01 of *M. ciceri* sv. biserrulae WSM1271 into the suicide vector pSacB. RepABC in pMESCI01 shares 100% identity with RepABC in pMc1192. To construct the curing plasmid-curing vector pMCC6, a 5,274-bp region containing the *repABC* region of pMESCI01 (Mesci_6410-6412) was PCR amplified with primers 13 and 14, containing 5' BamH1 and XbaI tails, and the region directionally cloned into pSacB, forming pMCC6. Plasmid pMCC6 was then transformed into ST18 and subsequently conjugated into CC1192 in a biparental mating performed in duplicate independently grown cultures of donor and recipient cells, and transconjugants were selected on G/RDM supplemented with neomycin with no added 5-aminolevulinic acid. Loss of plasmid pMc1192 was initially screened by PCR with primer pairs pr1 and pr2; pr3 and pr4; pr5 and pr6, each designed to amplify approximately 500-bp amplicons at three equidistant regions around pMc1192. Strains which did not yield expected products, consistent with the loss of pMc1192 were cured of pMCC6 by plating on RDM medium supplemented with 5% (w/v) sucrose and

counterselected for loss of neomycin resistance (encoded on pMCC6), producing MCC69 and MCC70, each derived from independent matings of CC1192 and ST18 (pMCC6) cells. To visualise curing of pMc11192, MCC69 and MCC70 were subjected to Eckhardt gel electrophoresis using a modified version of the procedure previously described (66). Briefly, the two plasmid cured derivatives, MCC69 and MCC70, wild type CC1192 and the reference strain Rlv3841 were grown in triplicate to optical densities OD_{600nm} of approximately 0.3 and a 200 µl aliquot of culture was then chilled on ice for 10 min before 1 ml of cold 0.3% (w/v) N-lauryl sarcosine in TBE buffer was added, mixed by inversion and incubated on ice for a further 10 min. The mixture was centrifuged (20,000 x g for 5 min at 4°C) and aspirated, before gentle resuspension in 25 µl lysis solution (0.1 mg ml^{-1} lysozyme, 10% (w/v) sucrose, 10 µg ml^{-1} RNase A in 1 x TBE). Immediately, 20 µl of the sample was loaded onto 0.75% (w/v) agarose with 1% (w/v) sodium dodecylsulfate and left for 30 min to settle, before electrophoresis at 70 V for 16 h at 4°C and subsequent UV visualisation after staining with ethidium bromide.

In vitro ICE transfer experiments

The ICE*Ml*Sym^{R7A}-devoid R7A derivative strain R7ANS harbours the broad-host range vector pPR3, encoding neomycin resistance, to facilitate selection against neomycin sensitive ICE-donor strains. R7ANS was mated with CC1192 as previously described (28) except that neomycin was substituted for tetracycline in selective media. PCR screening was carried out on a total of ten exconjugants selected from four independent mating experiments targeting the *ser*-tRNA (Meslo_RS0233700) with a 16-bp sequence at its 3' end identical to the core regions of ICE*Mc*Sym^{1192}, using primer pairs pr10 and pr11 (binding across the putative *attL* junction of the R7ANS chromosome and ICE*Mc*Sym^{1192}) and pr9 and pr12 (binding across the putative *attR* junction) (Table 3). Two of these exconjugants (MCC110 and MCC111) were selected for whole genome sequencing.

Sequencing, whole-genome assemblies and alignments

Sanger sequencing of PCR amplicons was performed by the Australian Genome Research Facility. For whole-genome sequencing, genomic DNA of plasmid-cured strain MCC69, R7ANS exconjugants MCC110 and MCC111, and field isolated strains WSM403 WSM4304, WSM4305, WSM4306, WSM4307, WSM4308, WSM4310, WSM4311, WSM4312, WSM4313 and WSM4315 was extracted using a Qiagen blood and tissue DNeasy extraction kit (catalogue #69054) according to the manufacturer's instructions. Concentration and purity were analysed by NanoDrop One (Thermo Scientific). Illumina MiSeq 2 x 250-bp paired-end reads (ACCESS Research Murdoch University) were used to produce draft genomes of all strains. *De novo* genome assemblies were performed using SPAdes version 3.10.1 software (67). Illumina sequencer adapter contamination was removed with neonsclip version 0.132 (https://github.com/Victorian-Bioinformatics-Consortium/nesoni) and reads were corrected using Lighter version 1.1.1 (68). Genomes were annotated using Prokka (69). NCBI accession numbers for assembled and annotated genomes can be found in Supplementary Table 1. For whole-genome BLASTN comparisons, Blast Ring Image Generator or BRIG (v0.9.5) (70) was used to produce BLASTN (options: -ungapped, -word_size 2000, upper and lower threshold 99%) alignments of sequence contigs or scaffolds of R7ANS exconjugant strains or field isolates with the complete genome of CC1192 (41).

Phylogenetic genome analysis was performed with bcgTree (71), which uses a concatenated alignment of 107 core genes conserved across bacterial genera to compare organisms. The tree was constructed using the RAxML (Randomized Axelerated Maximum Likelihood) program (72) with bootstraps set at 100. Phylogenetic and molecular evolutionary analyses of 16S rDNA sequences were performed using Geneious 11.1.5 (https://www.geneious.com) to construct a Neighbour Joining tree using the Tamura-Nei method to determine genetic distances with 5000 replications. Included in the analysis were field isolated *C. arietinum*-nodulating strains, WSM4303, WSM4304, WSM4305, WSM4306, WSM4307, WSM4308, WSM4310, WSM4311, WSM4312, WSM4313 and

406 WSM4315, along with CC1192, a selection of strains used as inoculants for other commercial legume
407 species, *Mesorhizobium* type strains and strains isolated from native Australian legume species. A list
408 of NCBI gene or genome accession numbers for each strain can be found in Table S2.

409 **Assessment of N_2 fixation with *C. arietinum* and statistical analysis**

410 *Cicer arietinum* cv Neelam was grown in free draining sterile sand in a glasshouse maintained at 22°C
411 as described by Yates, et al. (73), where growth of legumes is limited by N-deficiency except where
412 they are nodulated by N_2-fixing rhizobia. Briefly, seeds were surface-sterilized in 70% ethanol (1
413 min), followed by 4% NaOCl (3 min) and then rinsed in six successive washes of sterile deionised
414 water and imbibed in the final wash for 5 min. Surface-sterilised seeds were pre-germinated on 0.9%
415 (w/v) agar for several days at room temperature until emergence of the radicals and sown
416 aseptically into 1 L pots (170 x 80 x 80 mm) containing a coarse sand mix prepared as described by
417 Yates *et al.* (73), adjusted to pH 6.5 with a 5 g/L solution of $Fe_2(SO_4)_3$ prior to steam sterilizing. Each
418 pot was sown with two seedlings and thinned to one plant per pot upon emergence of shoots.

419 Cultures of rhizobia were incubated on ½LA plates (Hungria et al., 2016) for five days at 28°C and cell
420 growth suspended in 1% (w/v) sucrose at 10^8 cells ml^{-1}. *C. arietinum* treatment pots were either
421 uninoculated (N-starved and N-fed controls) or inoculated separately with 1 ml of the cell
422 suspension, while uninoculated pots received 1 ml of sterile deionised water. All pots were
423 protected from airborne contamination initially by plastic cling film, followed by sterile alkathene
424 beads as described by Yates *et al.* (2016). Each treatment consisted of five replications which were
425 randomized and maintained with sterile water and CRS plant growth nutrient solution (73) as
426 required. Uninoculated N-Fed control treatments received 5 ml of 0.1 M KNO_3 weekly.

427 Three separate glasshouse experiments were conducted to assess N_2 fixation of strains with *C.*
428 *arietinum*. Experiment (1) 3 evaluated the N_2 fixation effectiveness of 11 strains isolated from field-
429 cultivated *C. arietinum* growing in the northern grains belt of eastern Australia in an earlier study by
430 Elias and Herridge (37). These strains (WSM4303, WSM4304, WSM4305, WSM4306, WSM4307,

WSM4308, WSM4310, WSM4311, WSM4312, WSM4313 and WSM4315) had been previously shown to be different to the inoculant strain CC1192 by 16S rDNA sequencing and were isolated from paddock TA17 (near Moree), a site where *C. arietinum* had not been grown for 10 years. Experiment 2 measured the effectiveness of R7ANS exconjugant strains MCC110 and MCC111 harbouring ICE*Mc*Sym^{1192}, compared with R7A, R7ANS and wild-type CC1192. Experiment 1 (3) assessed the symbiotic phenotype of plasmid-cured derivatives of CC1192 against the wild-type strain.

To assess N_2 fixation effectiveness in Experiments 1 and 3, plants were harvested at 49 or 44 days post-inoculation, respectively, by carefully removing roots from soil and washing root systems. Nodules were excised from roots and plant shoots separated from roots at the hypocotyl, with both shoots and nodules then dried at 60°C until desiccated prior to weighing. For Experiment 2, assessment of nitrogenase activity was performed on intact plants prior to shoot and nodule biomass harvesting. Briefly, plants were harvested at 49 days post-inoculation by removing them carefully from pots and soil substrate and transferring them to 1000 mL Duran bottles with silicone septa. A total of 2% (v/v) acetylene was added to each bottle and the rate of acetylene reduction determined at 20°C as described by Yates and colleagues (73). Initial screening at time points of 1, 2 and 3 h indicated that the acetylene reduction relationship versus time was linear, thereafter samples were extracted at 2hr. Following the acetylene reduction assay, shoots and roots were harvested as described above. The variance of the means of the dry foliage weights, nodule weights and the various acetylene reduction assay parameters was assessed performing a one-way ANOVA and the significant difference between the treatment means analysed by the Tukey's HSD post-hoc test at alpha=0.05 using IBM SPSS Statistics version 24.

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456 **References**

457 1. Peoples MB, Brockwell J, Herridge DF, Rochester IJ, Alves BJR, Urquiaga S, Boddey RM,
458 Dakora FD, Bhattarai S, Maskey SL, Sampet C, Rerkasem B, Khan DF, Hauggaard-Nielsen H,
459 Jensen ES. 2009. The contributions of nitrogen-fixing crop legumes to the productivity of
460 agricultural systems. Symbiosis 48:1-17.
461 2. Herridge DF, Peoples MB, Boddey RM. 2008. Global inputs of biological nitrogen fixation in
462 agricultural systems. Plant Soil 311:1-18.
463 3. Vanlauwe B, Hungria M, Kanampiu F, Giller KE. 2019. The role of legumes in the sustainable
464 intensification of African smallholder agriculture: Lessons learnt and challenges for the
465 future. Agr Ecosys Environ 284:106583.
466 4. Davidson BR, Davidson HF. 1993. Legumes, the Australian experience: the botany, ecology
467 and agriculture of indigenous and immigrant legumes. Research Studies Press Ltd., Taunton.
468 5. Nichols PGH, Revell CK, Humphries AW, Howie JH, Hall EJ, Sandral GA, Ghamkhar K, Harris
469 CA. 2012. Temperate pasture legumes in Australia—their history, current use, and future
470 prospects Crop Pasture Sci 63:691-725.
471 6. Lange RT. 1961. Nodule bacteria associated with the indigenous Leguminosae of south-
472 western Australia. Microbiol 26:351-359.
473 7. Corbin E, Brockwell J, Gault R. 1977. Nodulation studies on chickpea (*Cicer arietinum*). Aust J
474 Exp Agr 17:126-134.
475 8. Howieson JG, Ewing MA, D'Antuono MF. 1988. Selection for acid tolerance in *Rhizobium*
476 *meliloti*. Plant Soil 105:179-188.
477 9. Howieson J, Loi A, Carr SJ. 1995. *Biserrula pelecinus* L. - a legume pasture species with
478 potential for acid, duplex soils which is nodulated by unique root-nodule bacteria. Aust J Agr
479 Res 46:997-1009.

480 10. Demezas DH, Reardon TB, Strain SR, Watson JM, Gibson AH. 1995. Diversity and genetic
481 structure of a natural population of *Rhizobium leguminosarum* bv. trifolii isolated from
482 *Trifolium subterraneum* L. Mol Ecol 4:209-220.
483 11. Hebb M, Richardson AE, Reid R, Brockwell J. 1998. PCR as an ecological tool to determine the
484 establishment and persistence of *Rhizobium* strains introduced into the field as seed
485 inoculant Aust J Exp Agr 49:923-934.
486 12. Ballard RA, Charman N, McInnes A, Davidson JA. 2004. Size, symbiotic effectiveness and
487 genetic diversity of field pea rhizobia (*Rhizobium leguminosarum* bv. viciae) populations in
488 South Australian soils. Soil Biol Biochem 36:1347-1355.
489 13. Stępkowski T, Moulin L, Krzyżańska A, McInnes A, Law IJ, Howieson J. 2005. European origin
490 of *Bradyrhizobium* populations infecting Lupins and *Serradella* in soils of Western Australia
491 and South Africa. Appl Environ Microb 71:7041-7052.
492 14. Ballard RA, Charman N. 2000. Nodulation and growth of pasture legumes with naturalised
493 soil rhizobia. 1. Annual *Medicago* Aust J Exp Agr 40:939-948.
494 15. Drew EA, Charman N, Dingemanse R, Hall E, Ballard RA. 2011. Symbiotic performance of
495 Mediterranean *Trifolium* spp. with naturalised soil rhizobia Crop Pasture Sci 62:903-913.
496 16. Gerding M, Howieson JG, O'Hara GW, Real D, Bräu L. 2013. Establishment and survival of the
497 South African legume *Lessertia* spp. and rhizobia in Western Australian agricultural systems.
498 Plant Soil 370:235-249.
499 17. Gerding M, O'Hara GW, Howieson JG, Bräu L. 2014. Overcoming non-selective nodulation of
500 *Lessertia* by soil-borne rhizobium in the presence of inoculant mesorhizobium. Plant Soil
501 380:117-132.
502 18. Nandasena KG, O'Hara GW, Tiwari RP, Sezmiş E, Howieson JG. 2007. *In situ* lateral transfer of
503 symbiosis islands results in rapid evolution of diverse competitive strains of mesorhizobia
504 suboptimal in symbiotic nitrogen fixation on the pasture legume *Biserrula pelecinus* L.
505 Environ Microbiol 9:2496-2511.

506 19. Mirkin BG, Fenner TI, Galperin MY, Koonin EV. 2003. Algorithms for computing parsimonious
507 evolutionary scenarios for genome evolution, the last universal common ancestor and
508 dominance of horizontal gene transfer in the evolution of prokaryotes. BMC Evol Biol 3:2.
509 20. Polz MF, Alm EJ, Hanage WP. 2013. Horizontal gene transfer and the evolution of bacterial
510 and archaeal population structure. Trends Genet 29:170-175.
511 21. Kloesges T, Popa O, Martin W, Dagan T. 2010. Networks of gene sharing among 329
512 proteobacterial genomes reveal differences in lateral gene transfer frequency at different
513 phylogenetic depths. Mol Biol Evol 28:1057-1074.
514 22. Perret X, Staehelin C, Broughton W. 2000. Molecular basis of symbiotic promiscuity.
515 Microbiol Mol Biol R 64:180 - 201.
516 23. Rubio LM, Ludden PW. 2008. Biosynthesis of the iron-molybdenum cofactor of nitrogenase.
517 Ann Rev Micro 62:93-111.
518 24. Remigi P, Zhu J, Young JPW, Masson-Boivin C. 2016. Symbiosis within symbiosis: evolving
519 nitrogen-fixing legume symbionts. Trends Microbiol 24:63-75.
520 25. Poole P, Ramachandran V, Terpolilli J. 2018. Rhizobia: from saprophytes to endosymbionts.
521 Nat Rev Microbiol 16:291-303.
522 26. Ramsay JP, Sullivan JT, Stuart GS, Lamont IL, Ronson CW. 2006. Excision and transfer of the
523 *Mesorhizobium loti* R7A symbiosis island requires an integrase IntS, a novel recombination
524 directionality factor RdfS, and a putative relaxase RlxS. Mol Microbiol 62:723-734.
525 27. Sullivan JT, Ronson CW. 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis
526 island that integrates into a phe-tRNA gene. Proc Natl Acad Sci U S A 95:5145-5149.
527 28. Haskett TL, Terpolilli JJ, Bekuma A, O'Hara GW, Sullivan JT, Wang P, Ronson CW, Ramsay JP.
528 2016. Assembly and transfer of tripartite integrative and conjugative genetic elements. Proc
529 Natl Acad Sci USA 113:12268-12273.
530 29. Sullivan JT, Trzebiatowski JR, Cruickshank RW, Gouzy J, Brown SD, Elliot RM, Fleetwood DJ,
531 McCallum NG, Rossbach U, Stuart GS, Weaver JE, Webby RJ, De Bruijn FJ, Ronson CW. 2002.

532 Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. J
533 Bacteriol 184:3086-3095.
534 30. Sullivan JT, Patrick HN, Lowther WL, Scott DB, Ronson CW. 1995. Nodulating strains of
535 *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. Proc
536 Natl Acad Sci U S A 92:8985-8989.
537 31. Ramsay J, Ronson CW. 2015. Genetic Regulation of Symbiosis Island Transfer in
538 *Mesorhizobium loti*, p 217-224. *In* De Bruijn FJ (ed), Biological Nitrogen Fixation
539 doi:10.1002/9781119053095. Ch21. John Wiley and Sons.
540 32. Howieson JG, Loi A, Carr SJ. 1995. *Biserrula pelecinus* L.- a legume pasture species with
541 potential for acid, duplex soils which is nodulated by unique root-nodule bacteria. Aust J Ag
542 Res 46:997-1009.
543 33. Nandasena KG, O'Hara GW, Tiwari RP, Howieson JG. 2006. Rapid *in situ* evolution of
544 nodulating strains for *Biserrula pelecinus* L. through lateral transfer of a symbiosis island
545 from the original mesorhizobial inoculant. Appl Environ Microb 72:7365-7367.
546 34. FAO. 2020. FAOSTAT: CROPS v15/6/20. http://www.fao.org/faostat/en/#data/QC.
547 35. Laranjo M, Alexandre A, Oliveira S. 2014. Legume growth-promoting rhizobia: An overview
548 on the *Mesorhizobium* genus. Microbiol Res 169:2-17.
549 36. Bullard GK, Roughley RJ, Pulsford DJ. 2005. The legume inoculant industry and inoculant
550 quality control in Australia: 1953–2003. Aust J Exp Agr 45:127-140.
551 37. Elias NV, Herridge DF. 2015. Naturalised populations of mesorhizobia in chickpea (*Cicer*
552 *arietinum* L.) cropping soils: effects on nodule occupancy and productivity of commercial
553 chickpea. Plant Soil 387:233-249.
554 38. Greenlon A, Chang PL, Damtew ZM, Muleta A, Carrasquilla-Garcia N, Kim D, Nguyen HP,
555 Suryawanshi V, Krieg CP, Yadav SK, Patel JS, Mukherjee A, Udupa S, Benjelloun I, Thami-
556 Alami I, Yasin M, Patil B, Singh S, Sarma BK, von Wettberg EJB, Kahraman A, Bukun B, Assefa
557 F, Tesfaye K, Fikre A, Cook DR. 2019. Global-level population genomics reveals differential

effects of geography and phylogeny on horizontal gene transfer in soil bacteria. Proc Natl Acad Sci USA 116:15200-15209.

39. Lafay B, Burdon JJ. 1998. Molecular diversity of rhizobia occurring on native shrubby legumes in southeastern Australia. Appl Environ Microb 64:3989-3997.

40. Hoque MS, Broadhurst LM, Thrall PH. 2011. Genetic characterization of root-nodule bacteria associated with *Acacia salicina* and *A. stenophylla* (Mimosaceae) across south-eastern Australia. Int J Syst Evol Micr 61:299-309.

41. Haskett T, Wang P, Ramsay J, O'Hara G, Reeve W, Howieson J, Terpolilli J. 2016. Complete genome sequence of *Mesorhizobium ciceri* strain CC1192, an efficient nitrogen-fixing microsymbiont of *Cicer arietinum*. Genome Announc 4.

42. Cabanes D, Boistard P, Batut J. 2000. Identification of *Sinorhizobium meliloti* genes regulated during symbiosis. J Bacteriol 182:3632-3637.

43. Ramsay JP, Major AS, Komarovsky VM, Sullivan JT, Dy RL, Hynes MF, Salmond GPC, Ronson CW. 2013. A widely conserved molecular switch controls quorum sensing and symbiosis island transfer in *Mesorhizobium loti* through expression of a novel antiactivator. Mol Microbiol 87:1-13.

44. Haskett TL, Terpolilli JJ, Ramachandran VK, Verdonk CJ, Poole PS, O'Hara GW, Ramsay JP. 2018. Sequential induction of three recombination directionality factors directs assembly of tripartite integrative and conjugative elements. PLOS Genet 14:e1007292.

45. Preisig O, Zufferey R, Hennecke H. 1996. The *Bradyrhizobium japonicum fixGHIS* genes are required for the formation of the high-affinity cbb_3-type cytochrome oxidase. Arch Microbiol 165:297-305.

46. Preisig O, Zufferey R, Thöny-Meyer L, Appleby CA, Hennecke H. 1996. A high-affinity cbb_3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. J Bacteriol 178:1532-1538.

47. Kahn D, David M, Domergue O, Daveran ML, Ghai J, Hirsch PR, Batut J. 1989. *Rhizobium meliloti fixGHI* sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. J Bacteriol 171:929-939.

48. Mandon K, Kaminski PA, Mougel C, Desnoues N, Dreyfus B, Elmerich C. 1993. Role of the *fixGHI* region of *Azorhizobium caulinodans* in free-living and symbiotic nitrogen fixation. FEMS Microbiol Lett 114:185-189.

49. Roh JH, Kaplan S. 2000. Genetic and phenotypic analyses of the *rdx* locus of *Rhodobacter sphaeroides* 2.4.1. J Bacteriol 182:3475-3481.

50. Koch H-G, Winterstein C, Saribas AS, Alben JO, Daldal F. 2000. Roles of the *ccoGHIS* gene products in the biogenesis of the cbb_3-type cytochrome c oxidase. J Mol Biol 297:49-65.

51. Brewer RJM, Haskett TL, Ramsay JP, O'Hara GW, Terpolilli JJ. 2017. Complete genome sequence of *Mesorhizobium ciceri* bv. biserrulae WSM1497, an efficient nitrogen-fixing microsymbiont of the forage legume *Biserrula pelecinus*. Genome Announc 5:e00902-17.

52. Nandasena K, Yates R, Tiwari R, O'Hara G, Howieson J, Ninawi M, Chertkov O, Detter C, Tapia R, Han S, Woyke T, Pitluck S, Nolan M, Land M, Liolios K, Pati A, Copeland A, Kyrpides N, Ivanova N, Goodwin L, Meenakshi U, Reeve W. 2014. Complete genome sequence of *Mesorhizobium ciceri* bv. biserrulae type strain (WSM1271^T). Stand Genomic Sci 9:462-72.

53. Nandasena KG, O'Hara G W, Tiwari RP, Howieson JG. 2006. Rapid in situ evolution of nodulating strains for Biserrula pelecinus L. through lateral transfer of a symbiosis island from the original mesorhizobial inoculant. Appl Environ Microbiol 72:7365-7.

54. Lafay B, Burdon JJ. 2001. Small-Subunit rRNA genotyping of rhizobia nodulating Australian *Acacia* spp. Appl Environ Microb 67:396-402.

55. Lafay B, Burdon JJ. 2007. Molecular diversity of legume root-nodule bacteria in Kakadu National Park, Northern Territory, Australia. PLOS ONE 2:(3): e277.

607 56. Thrall PH, Burdon JJ, Woods MJ. 2000. Variation in the effectiveness of symbiotic
608 associations between native rhizobia and temperate Australian legumes: interactions within
609 and between genera. J Appl Ecol 37:52-65.
610 57. Marsudi NDS, Glenn AR, Dilworth MJ. 1999. Identification and characterization of fast- and
611 slow-growing root nodule bacteria from South-Western Australian soils able to nodulate
612 *Acacia saligna*. Soil Biol Biochem 31:1229-1238.
613 58. Sullivan JT, Eardly BD, van Berkum P, Ronson CW. 1996. Four unnamed species of
614 nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. Appl Environ
615 Microb 62:2818-2825.
616 59. Reeve W, Nandasena K, Yates R, Tiwari R, Hara G, Ninawi M, Gu W, Goodwin L, Detter C,
617 Tapia R, Han C, Copeland A, Liolios K, Chen A, Markowitz V, Pati A, Mavromatis K, Woyke T,
618 Kyrpides N, Ivanova N, Howieson J. 2013. Complete genome sequence of *Mesorhizobium*
619 *australicum* type strain WSM2073^{T}. Stand Genomic Sci 9:410-419.
620 60. Reeve W, Nandasena K, Yates R, Tiwari R, O'Hara G, Ninawi M, Chertkov O, Goodwin L, Bruce
621 D, Detter C, Tapia R, Han S, Woyke T, Pitluck S, Nolan M, Land M, Copeland A, Liolios K, Pati
622 A, Mavromatis K, Markowitz V, Kyrpides N, Ivanova N, Meenakshi U, Howieson J. 2013.
623 Complete genome sequence of *Mesorhizobium opportunistum* type strain WSM2075^{T}. Stand
624 Genomic Sci 9:294-303.
625 61. Hungria M, O'Hara GW, Zilli JE, Araujo RS, Deaker R, Howieson JG. 2016. Isolation and
626 growth of rhizobia, p 39-60. *In* Howieson JG, Dilworth MJ (ed), Working with rhizobia. ACIAR.
627 62. Howieson JG, Ewing MA. 1986. Acid tolerance in the *Rhizobium meliloti-Medicago* symbiosis.
628 Aust J Ag Res 37:55-64.
629 63. Beringer JE. 1974. R factor transfer in *Rhizobium leguminosarum*. J Gen Microbiol 84:188-98.
630 64. Ronson CW, Primrose SB. 1979. Carbohydrate metabolism in *Rhizobium trifolii:* identification
631 and symbiotic properties of mutants J Gen Microbiol:77-88.

632 65. Bertani G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic
633 *Escherichia coli*. J Bacteriol 62:293-300.
634 66. Eckhardt T. 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in
635 bacteria. Plasmid 1:584-588.
636 67. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI,
637 Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner
638 PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell
639 sequencing. J Comp Biol 19:455-477.
640 68. Song L, Florea L, Langmead B. 2014. Lighter: fast and memory-efficient sequencing error
641 correction without counting. Genome Biol 15:509.
642 69. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068-
643 2069.
644 70. Alikhan N-F, Petty NK, Ben Zakour NL, Beatson SA. 2011. BLAST Ring Image Generator (BRIG):
645 simple prokaryote genome comparisons. BMC Genomics 12:402.
646 71. Ankenbrand MJ, Keller A. 2016. bcgTree: automatized phylogenetic tree building from
647 bacterial core genomes. Genome 59:783-791.
648 72. Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of
649 large phylogenies. Bioinformatics 30:1312-1313.
650 73. Yates RJ, Howieson JG, Hungria M, Bala A, O'Hara GW, Terpolilli JJ. 2016. Chapter 5:
651 Authentication of rhizobia and assessment of the legume symbiosis in controlled plant
652 growth systems. *In* Howieson JG, Dilworth MJ (ed), Working with rhizobia. Australian Centre
653 for International Agricultural Research, Canberra.
654 74. Johnston AWB, Beringer JE. 1975. Identification of the *Rhizobium* strains in pea root nodules
655 using genetic markers. J Gen Microbiol 87:343-350.
656 75. Thoma S, Schobert M. 2009. An improved *Escherichia coli* donor strain for diparental mating.
657 FEMS Micro Lett 294:127-132.

76. Prentki P, Krisch HM. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene 29:303-13.

77. Quandt J, Hynes MF. 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene 127:15-21.

78. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A 76:1648-52.

79. Rodpothong P, Sullivan JT, Songsrirote K, Sumpton D, Cheung KWJ-T, Thomas-Oates J, Radutoiu S, Stougaard J, Ronson CW. 2009. Nodulation gene mutants of *Mesorhizobium loti* R7A—*nodZ* and *nolL* mutants have host-specific phenotypes on *Lotus* spp. Mol Plant-Micro Int 22:1546-1554.

679 **Table 1** : Acetylene reduction assay of *C. arietinum* cv. Neelam grown for 49 days and inoculated
680 separately with R7ANS (ICE*Mc*Sym[1192]) exconjugants MCC110, MCC111, or CC1192. Treatment
681 means are shown ± standard errors. A one-way ANOVA detected no significant difference ($P \leq 0.05$)
682 between the treatments.

Strain	ARA per plant (µmol acetylene reduced/plant/h)	ARA per nodule (nmol acetylene reduced/nodule/h)	ARA per nodule mass (nmol acetylene reduced/mg nodule/h)
CC1192	1.020 ± 0.255	32.2 ± 9.1	13.4 ± 2.9
MCC110	0.827 ± 0.259	18.8 ± 5.1	7.90 ± 2.0
MCC111	1.146 ± 0.082	25.2 ± 2.6	12.3 ± 2.1

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695 **Table 2**: Strains and plasmids used in this study

Strain or Plasmid	Genotype or Alternative name	Reference
Strains		
CC1192	Wild-type *Mesorhizobium ciceri* sv. ciceri CC1192, harbouring ICE*Mc*Sym^{1192}	(7)
R7A	Wild-type *Mesorhizobium japonicum* field isolate of ICMP 3153, harbouring ICE*Ml*Sym^{R7A}.	(30)
R7ANS	Non-symbiotic derivative of R7A; lacks ICE*Ml*Sym^{R7A}, harbouring BHR vector pPR3, Nm^{R}	(26)
MCC69	CC1192 derivative, cured of plasmid pMc1192	This study
MCC70	CC1192 derivative, cured of plasmid pMc1192	This study
MCC110	R7ANS exconjugant carrying ICE*Mc*Sym^{1192} integrated at *ser*-tRNA	This study
MCC111	R7ANS exconjugant carrying ICE*Mc*Sym^{1192} integrated at *ser*-tRNA	This study
WSM4303	Nat2 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
WSM4304	Nat3 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
WSM4305	Nat4 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
WSM4306	Nat5 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
WSM4307	Nat7 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
WSM4308	Nat8 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
WSM4310	Nat18 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
WSM4311	Nat19 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
WSM4312	Nat20 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
WSM4313	Nat21 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
WSM4315	Nat28 from field- cultivated *C. arietinum*: *Mesorhizobium sp.*	(37)
Rlv3841	Wild-type *Rhizobium leguminosarum* sv. viciae 3841	(74)
DH5α	*Escherichia coli* strain for cloning: F^{-} *ϕ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17*(r_k^-, m_k^+) *phoA supE44 thi-1gyrA96 relA1*	Invitrogen
ST18	*Escherichia coli*: S17-1Δ*hemA thi pro hsdR-M-* with chromosomal integrated [RP4-2 Tc::Mu:Km^{R}::Tn*7*, Tra^{+} Tri^{+R} Sm^{R}]	(75)
Plasmids		
pJET1.2/Blunt	PCR product cloning vector; Ap^{r}	Thermo-Fisher
pHP45-ΩSmSp	pHP derivative with ΩSmSp cassette, Sm^{r} Sp^{r}	(76)
pJQ200SK	pACYC derivative, P15A origin of replication insertional mutagenesis inactivation vector, Gm^{r} Suc^{s}	(77)
pRK2013	Helper plasmid used for mobilizing plasmids. ColE1 replicon with RK2 *tra* genes, Km^{R}	(78)
pMCC6	pSacB carrying 5,274-bp *repABC* region from *M. ciceri* sv. WSM1271	This study
pSacB	BHR vector carrying inducible IPTG promoter and *sacB* gene, Nm^{R}, Suc^{S}.	(28)
pPR3	BHR pPROBE-KT carrying the *nptII* promoter from pFAJ1708, Nm^{R}	(79)

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698 **Table 3:** Primers used in this study

Primer number	Name	Oligonucleotide sequence (5'-3')	Replicon specificity
pr1	pMc1192-Fa	CGTTCGGACTTGAACCAGGA	pMc1192
pr2	pMc1192-Ra	CCTCAAAGCTGGCATCGAAC	pMc1192
pr3	pMc1192-Fb	GATCAATGGTGCGCGAGAAC	pMc1192
pr4	pMc1192-Rb	CGCTGTTTCGACGGTTTGTT	pMc1192
pr5	pMc1192-Fc	TTCCCCGAACGAGATTGCAA	pMc1192
pr6	pMc1192-Rc	AAGCGCGATCGACAGATGAT	pMc1192
pr7	CC1192_attB_F	GTTGTCGGGACTGTTGTTGG	CC1192 chromosome
pr8	CC1192_attB_R	TTGGTTTCTCCTCGAAGCGG	CC1192 chromosome
pr9	CC1192_attP_F	GCCGATTGTCACAGGCTACT	ICEMcSym[1192]
pr10	CC1192_attP_R	CGGACGAGATACCAGATGCC	ICEMcSym[1192]
pr11	R7ANS_attB_F	GTTATTGGCCGGCAAAGACC	R7ANS serine
pr12	R7ANS_attB_R	TTTCCGACCTACACGCTCAC	R7ANS serine
pr13	RepABC F BamHI	ATCAGGGATCCGTTGACCTCCGCATGCAAAC	This study
pr14	RepABC R XbaI	ATCAG TCTAGAGTCAATCTCACCAGGGCCAG	This study

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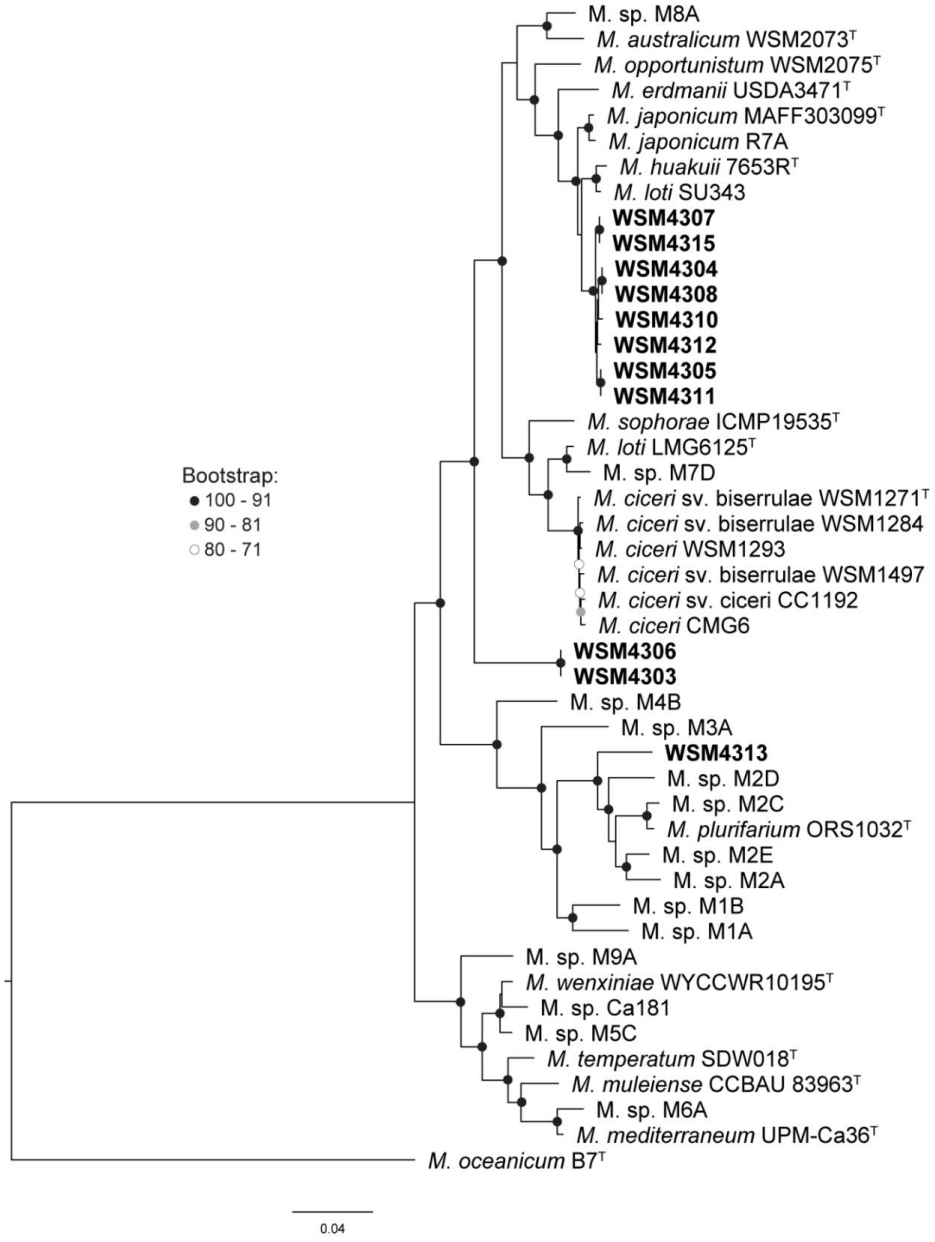
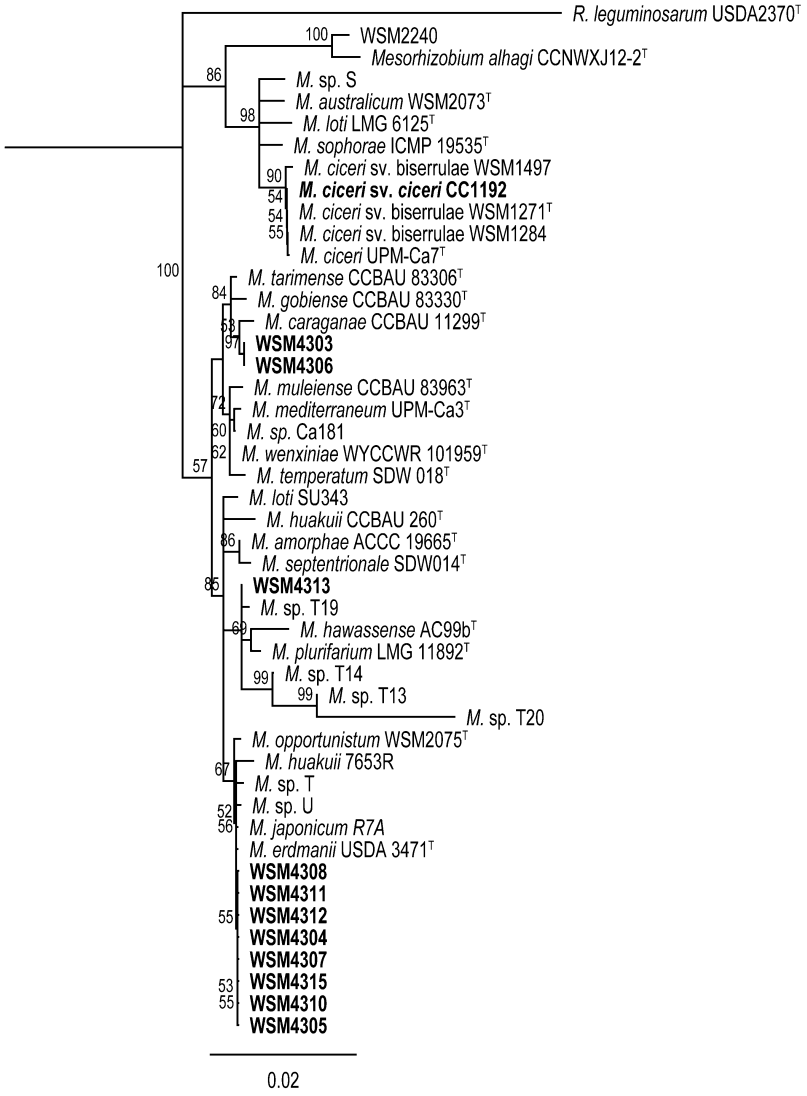


Figure 1: Genome tree of *Mesorhizobium* isolates performed with bcgTree using a concatenated alignment of 107 core proteins conserved across bacterial genera to compare organisms. The tree was constructed using the RAxML (Randomized Axelerated Maximum Likelihood) program with 100 bootstraps. In this tree, the genome of *M. ciceri* Ca181 has been renamed M. sp Ca181 due to being genetically distinct from the *M. ciceri* clade.



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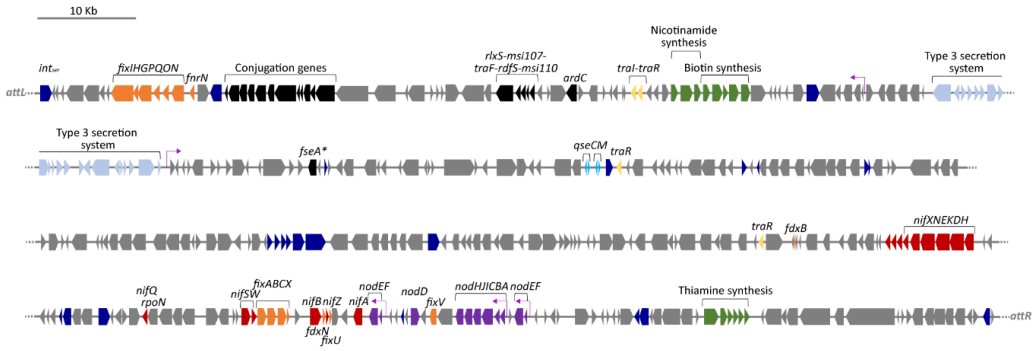
707 **Figure 2:** Neighbour joining phylogenetic tree of 16S rDNA sequences of *Mesorhizobium* isolates
708 from this study and other RNB obtained from GenBank® (type strains indicated by ^{T} adjacent to
709 strain identification). Isolates from this study and the commercial inoculant *M. ciceri* CC1192 are
710 indicated in bold type. Analyses were conducted in Geneious 11.1.5 using the Tamura-Nei method to
711 determine genetic distances with *Rhizobium leguminosarum* bv. *viciae* USDA 2370^{T} as the outgroup.
712 Branch values less than 50% are not shown from 5000 bootstraps.



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714 **Figure 3. Structure of ICE*Mc*Sym^{CC1192}.** *M. ciceri* CC1192 carries a 418,907 bp symbiosis ICE (base pairs 4,216,431 to 4,635,338) adjacent to the sole *ser*-tRNA
715 (A4R28_RS20655) in the *M. ciceri* CC1192 genome, with the ICE containing 373 predicted coding sequences. Gene annotations are colour coded as follows:
716 Mobile genes such as transposases, integrases and recombinases (blue); Type 3 secretion systems (T3SS; pale blue); ICE transfer genes (black); quorum
717 sensing genes (yellow); vitamin biosynthesis (green); nitrogen-fixation genes, *nif* (red) and *fix* (orange), and nodulation genes, *nod* (purple). Putative *nod*
718 boxes are indicated by arrows. Hypothetical protein is indicated by hp, (°) indicates pseudogene and (*) shows frameshifting is required for gene to be
719 translated. Gene coordinates are provided in Table S1.

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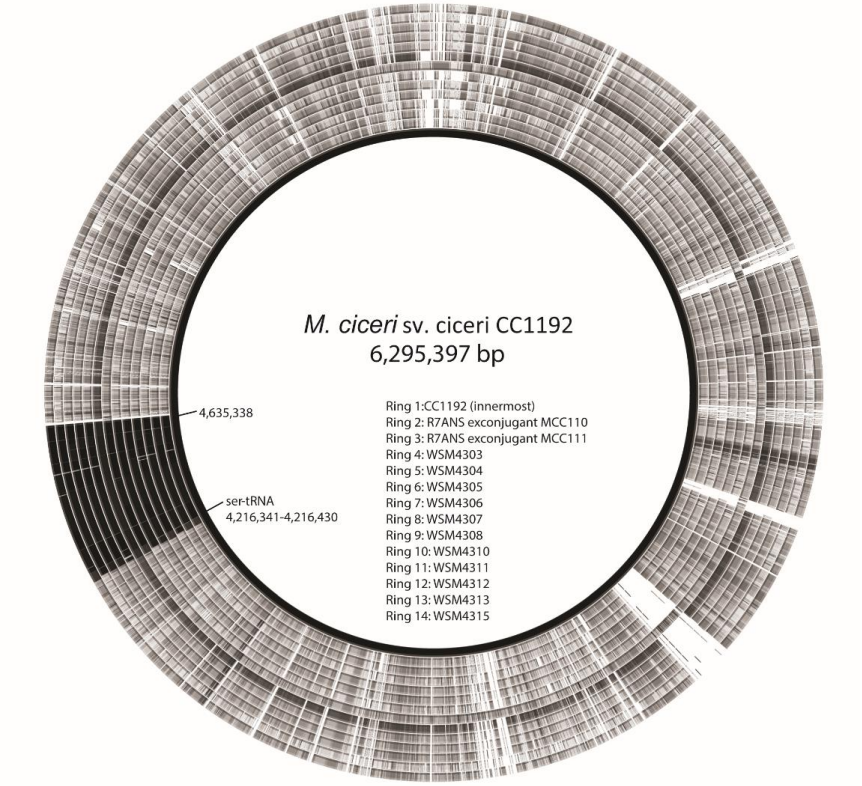

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722 **Figure 4**: Conservation of ICE*Mc*Sym[1192] in CC1192 (inner circle), two exconjugants of R7ANS
723 (MCC110 and MCC111) and eleven novel *C. arietinum*-nodulating field isolates, compared to the
724 reference genome of CC1192 . Circular BLASTN alignments carried out using BRIG (70) of CC1192
725 with MCC110, MCC111, WSM4303, WSM4304, WSM4305, WSM4306, WSM4307, WSM4308,
726 WSM4310, WSM4311, WSM4312, WSM4313 and WSM4315. Black regions indicate >99% conserved
727 nucleotide identity.

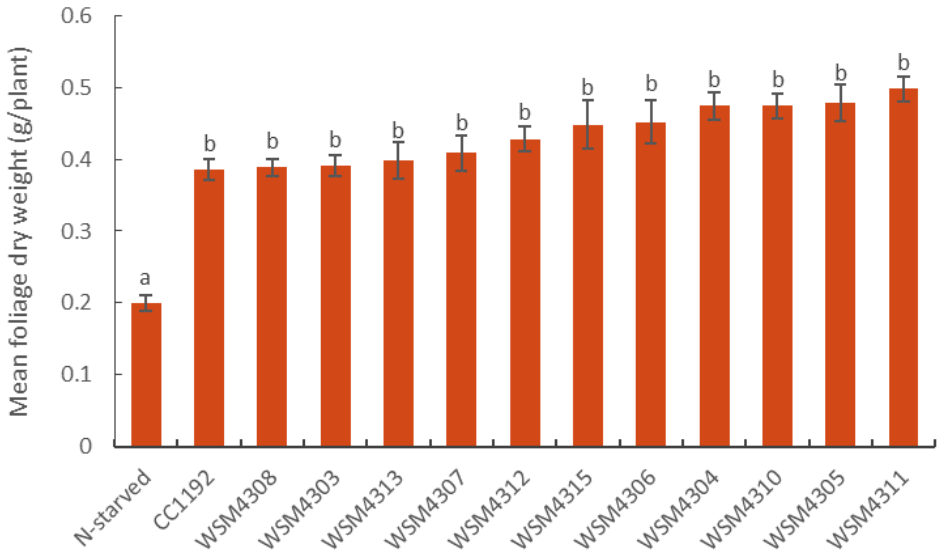

Figure 5: Mean foliage dry weight of *Cicer arietinum* cv. Neelam inoculated sepeartely with 11 novel isolates and the commercial inoculant *M. ciceri* CC1192 and grown for 49 days. Treatments are shown with standard error of means and those treaments that share a letter are not significantly different according to the Tukey HSD test ($P \leq 0.05$). Uninoculated control plants received no nitrogen (N-starved) and were devoid of nodules.

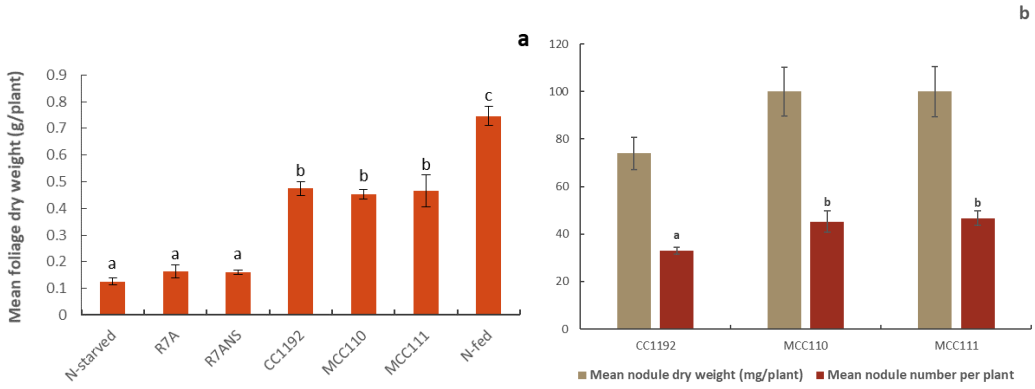

Figure 6: Symbiotic effectiveness of exconjugants of the ICE*Mc*Sym^{1192} mobilsed into R7ANS (MCC110 and MCC111) compared to wild type CC1192, R7A (*Mesorhizobium japonicum* strain carrying ICE*Mj*Sym^{R7A}) and R7ANS (symbiosis ICE-devoid derivative of R7A) on *C. aritetinum* cv. Neelam. a) Mean folaige dry weight and b) Mean dry nodule weight per plant and nodule number of plants grown in nitrogen-limited conditions and inoculated separately with indicated strains, grown for 49 days. Uninoculated and N-fed (supplied as KNO_3) plants were included as negative and positive controls, respectively. Treatments are shown with standard error of means and those treaments that share a letter are not significantly different according to the Tukey HSD test ($P \leq 0.05$). A one-way ANOVA detected no significant difference ($P \leq 0.05$) between the treatments for nodule dry weights.

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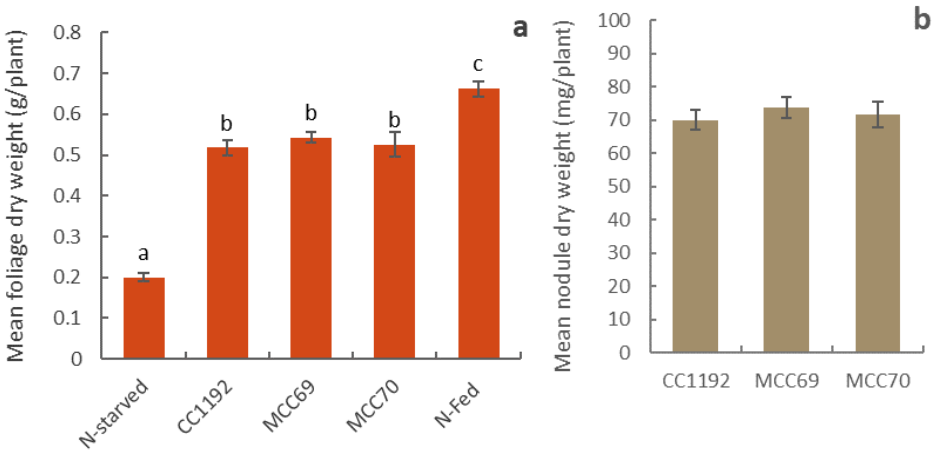

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752 **Figure 7:** Symbiotic effectiveness of plasmid-cured derivatives of CC1192 (MCC69 and MCC70) compared to CC1192 on *C. arietinum* cv. Neelam. (a) Mean
753 foliage dry weight and (b) Mean nodule dry weight per plant of *C. arietinum* inoculated separately with indicated strains and grown free of added nitrogen
754 in controlled glasshouse conditions for 44 days. Uninoculated control plants received no nitrogen (N-starved) or nitrogen as KNO_3 (N-fed) and roots of these
755 plants were devoid of nodules at harvest. Error bars represent standard error of means and treaments that share a letter are not significantly different,
756 according to the Tukey HSD test ($P \leq 0.05$). A one-way ANOVA detected no significant difference ($P \leq 0.05$) between the treatments for nodule dry weights.