

1 **TITLE**

2 **Massive rhizobial genomic variations associated with partner quality in *Lotus*–**

3 ***Mesorhizobium* symbiosis**

4

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27 **KEYWORDS**

28 Legume–rhizobia mutualism, whole-genome sequencing, partner quality variation,

29 horizontal gene transfer

30 **ABSTRACT**

31 In diverse mutualistic relationships, genetic variations in impact on the growth of
32 interacting partners—variations in partner quality—are common, despite the theoretical
33 prediction that selection favoring high-quality partners should eliminate such variations.
34 Here, we investigated how variations in partner quality could be maintained in the
35 nitrogen-fixing mutualism between *Lotus japonicus* and *Mesorhizobium* bacteria. We
36 reconstructed *de novo* assembled full-genome sequences from nine rhizobial symbionts,
37 finding massive variations in the core genome and the contrastingly similar symbiotic
38 islands, indicating recent horizontal gene transfer (HGT) of the symbiosis islands into
39 diverse *Mesorhizobium* lineages. A cross-inoculation experiment using nine sequenced
40 rhizobial symbionts and 15 *L. japonicus* accessions revealed extensive quality variations
41 represented by plant growth phenotypes, including genotype-by-genotype interactions.
42 Quality variations were not associated with the presence/absence variations of known
43 symbiosis-related genes in the symbiosis island, but rather, showed significant
44 correlations with the core genome variations, supported by SNP- and kinship matrix-
45 based association analyses. These findings highlight the novel role of HGT of symbiosis
46 islands, which indirectly supply mutations of core genomes into *L. japonicus*-associated
47 bacteria, thereby contributing to the maintenance of variations in partner quality.

48 INTRODUCTION

49 While mutualistic symbiotic relationships—unlike organisms living together and
50 establishing a cooperative interaction—are ubiquitous in nature, it remains unclear how
51 mutualistic interactions originated and how they are evolutionarily maintained.
52 Evolutionary theory predicts that mutualism might be unstable because natural selection
53 would favor mutualists that optimize their fitness by minimizing the costs of returning
54 benefits to a partner (Frederickson 2013; Ghoul *et al.* 2013). Therefore, the stabilizing
55 mechanisms that prevent the invasion of low-quality partners have been a major focus
56 of studies (Heath and Stinchcombe 2014), such as those involving partner choice (Bull
57 and Rice 1991), partner fidelity feedback (Bull and Rice 1991; Archetti *et al.* 2011) and
58 sanctions against such invasion (Kiers *et al.* 2003).

59 Although such stabilizing mechanisms should reduce variations in partner
60 quality in mutualistic symbiosis, thereby maintaining high-quality mutualists, variations
61 in partner quality have been observed in diverse mutualistic relationships (Thrall *et al.*
62 2000; Sachs *et al.* 2010;). Multiple models have been proposed to resolve this
63 discrepancy, such as mutation–selection balance and spatiotemporally varying selection
64 (Van Dyken *et al.* 2011; Simonsen and Stinchcombe 2014; Smith *et al.* 2014; Steidinger
65 and Bever 2014; reviewed by Heath and Stinchcombe 2014). The mutation–selection
66 balance is a model postulating that low-quality partners evolve via mutations but are
67 slowly purged from populations by purifying selection. The model of spatiotemporally
68 varying selection predicts that the fitness of a partner’s genotype varies depending on
69 the genotype of an interacting species, such as genotype \times genotype ($G \times G$)
70 interactions, on the spatially variable environment conditions ($G \times E$ interactions) or on
71 temporally variable selection (Denison and Kiers 2004). While these models provide

72 possible explanations for the persistence of variations in partner quality in mutualist
73 relationships, it remains unclear how variations in partner quality arise and how they are
74 maintained in natural populations.

75 The legume–rhizobia mutualism is an ideal model to address this question
76 because we can manipulate genotypes of both species and reconstruct their interactions
77 *in vitro*. To understand the origin and the maintenance of variations in partner quality at
78 the microevolutionary scale, it is essential to quantify the quality variations among
79 strains originated from single leguminous species and disentangle the genetic basis
80 underlying the rhizobial quality. Although variations in rhizobial quality have been
81 observed in multiple systems of legume–rhizobia mutualisms (e.g. *Acacia–Ensifer*,
82 Barrett *et al.* 2015 and 2016; *Medicago–Ensifer*, Porter *et al.* 2011), most previous
83 studies were based on a limited number of genes, and thus, the genetic basis underlying
84 the rhizobial quality was unclear. Although Porter *et al.* (2019) and Klinger *et al.* (2016)
85 analyzed the genetic basis of the rhizobial quality variation using genome-wide
86 polymorphism data, these studies used a limited number of plant strains, and therefore,
87 did not take $G \times G$ interactions into account. Furthermore, genome-wide polymorphism
88 data of these previous studies were based on resequencing using Illumina short-reads,
89 making it difficult to investigate structural variations suggested to be important for
90 plant–microbe interactions (Raffaele *et al.* 2010; Tsushima *et al.* 2019).

91 Here, we focused on the mutualism between *Lotus japonicus* (Regel) K. Larsen
92 and their rhizobial symbionts. *Lotus japonicus* has been regarded as a model species for
93 the understanding of plant–microbe interactions (Bamba *et al.* 2019a), and there have
94 been extensive studies on the molecular, physiological and genomic bases of plant–
95 rhizobia symbiosis (Handberg and Stougaard 1992; Szczyglowski *et al.* 1998; Kouchi *et*

96 *al.* 2004; Maekawa *et al.* 2009; Madsen *et al.* 2010; Suzuki *et al.* 2011; Soyano *et al.*
97 2013; Nishida *et al.* 2016, 2018). Among *L. japonicus*-associated symbionts, full-
98 genome sequence information is available from two strains, *Mesorhizobium japonicum*
99 MAFF303099 and *M. loti* TONO (Kaneko *et al.* 2000; Shimoda *et al.* 2016). Bamba *et*
100 *al.* (2019b) explored the genetic diversity of *L. japonicus*-associated symbionts, finding
101 that *L. japonicus* in natural populations were associated with highly diverse
102 *Mesorhizobium* bacteria. However, they used only three housekeeping and five
103 symbiotic genes, and the detailed genomic variations of *L. japonicus*-associated
104 symbionts are still unknown. In this study, to investigate the genomic variations of *L.*
105 *japonicus*-associated symbionts, we first reconstructed high quality *de novo* assembled
106 genome sequences from nine rhizobial symbionts sampled from three geographically
107 distinct locations in Japan. Second, to quantify the rhizobial variations in partner quality
108 including G × G interactions, we performed a cross-inoculation experiment using nine
109 full-genome sequenced rhizobial symbionts and 15 *L. japonicus* natural accessions.
110 Three of those *L. japonicus* accessions originated from the same locations where the
111 nine rhizobial strains were collected, so we could explore a signature of local
112 adaptation: i.e. native rhizobial genotypes outperform foreign rhizobial genotypes when
113 associated with the host genotypes originating from the same locations. Finally, to infer
114 which genomic regions were responsible for rhizobial variations in partner quality, we
115 performed a series of analyses testing the association between genomic polymorphisms
116 and rhizobial variations in partner quality.

117 MATERIALS AND METHODS

118 Bacterial strains

119 We used nine *L. japonicus*-associated *Mesorhizobium* strains for this study, which were
120 previously referred to as 113-1-1, 113-3-3, 113-3-9, 131-2-1, 131-2-5, 131-3-5, L-2-11,
121 L-8-3 and L-8-10 (Bamba *et al.* 2019b). These nine strains were sampled from three
122 geographically distinct localities, Tottori (113-1-1, 113-3-3 and 113-3-9), Aomori (131-
123 2-1, 131-2-5 and 131-3-5) and Miyakojima (L-2-11, L-8-3 and L-8-10) (Supporting
124 Information Table S1), where *L. japonicus* natural accessions of the Natural
125 BioResource Project also originated (MG50 from Tottori, MG23 from Aomori and
126 MG20 from Miyakojima; Supporting Information Table S2). Details of the sampling
127 localities have been described by Bamba *et al.* (2019b).

128

129 DNA extraction and whole-genome sequencing using MinION and Illumina HiSeq

130 Prior to DNA extraction, we cultured rhizobial strains on a tryptone yeast (TY) agar
131 plate for 4 days at 28°C, and then picked single colonies and incubated them for 3 days
132 at 28°C in liquid TY medium. After incubation, we precipitated the cells by
133 centrifugation at 13,000 *g* for 3 min and rinsed them with sterilized MilliQ water
134 (Millipore Corp., Burlington, MA, USA) twice. The genomic DNA of each rhizobial
135 strain was extracted using a NucleoBond CB20 system (MACHERY-NAGEL GmbH &
136 Co. KG, Düren, Germany), according to the manufacturer's instructions. The quality of
137 genomic DNA was confirmed using agarose gel electrophoresis and a BioSpec-nano
138 system (Shimadzu, Kyoto, Japan).

139 We performed whole-genome sequencing analyses using Oxford Nanopore
140 Technologies (ONT) MinION and Illumina HiSeq 2500 systems. The library for ONT
141 MinION was prepared using Rapid Barcoding kits (SQK-RBK004). We adjusted all
142 nine libraries to the same concentration, mixed them together and then loaded them onto
143 R9.4 flow cells. The sequencing run was performed twice on a MinION MK1b device
144 following the NC_48h_Sequencing_Run_FLO-MIN106_SQK-RBK004 protocol. The
145 library preparation for Illumina HiSeq and sequencing run were performed by
146 DNAFORM (RIKEN, Yokohama, Japan).

147

148 **Preprocessing of next-generation sequencing data, *de novo* assembly and**
149 **annotation**

150 The ONT reads were demultiplexed with Albacore 2.2.2
151 (<https://github.com/Albacore/albacore>), and adapter sequences were trimmed with
152 Porechop 0.2.3 (<https://github.com/rrwick/Porechop>). The quality of demultiplexed
153 reads was calculated with NanoStat 1.1.0 (De Coster *et al.* 2018). The ONT reads were
154 processed with NanoFilt 2.2.0 (De Coster *et al.* 2018) to keep sequences with a q-score
155 > 8, and the first 100 bases were removed to increase sequence quality, with a minimum
156 sequence length of 1 kb.

157 The overall quality of the HiSeq reads was evaluated using FastQC
158 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). After confirming the lack
159 of technical errors in the sequencing, low-quality tails were trimmed from each read
160 using SolexaQA (Cox *et al.* 2010) with a cutoff threshold set at a q-score of 30, and
161 reads shorter than 75 bp were filtered with PRINSEQ++ (Schmieder and Edwards,

162 2011). The Hiseq-filtered reads with pair-end relationships were repaired with BBtools
163 (<https://sourceforge.net/projects/bbmap/>).

164 The hybrid read set (both Illumina and ONT reads) for each isolate was
165 assembled using Unicycler 0.4.0 (Wick *et al.* 2017) in its conservative mode. Unicycler
166 performs the assembly of the Illumina reads with SPAdes 3.12.0 (Bankevich *et al.*
167 2012), and assembly graph scaffolds were then prepared using ONT reads. Unicycler
168 was used to polish the final assembly of Illumina reads, and Pilon (Walker *et al.* 2014)
169 was applied to reduce the rate of small base-level errors. The resulting assembly graph
170 was visualized using Bandage (Wick *et al.* 2015).

171 The assembled genomes were annotated with the Rapid Annotation using
172 Subsystem Technology (RAST) annotation server (<http://rast.theseed.org/FIG/rast.cgi>;
173 Aziz *et al.* 2008; Brettin *et al.* 2015; Overbeek *et al.* 2014). Annotation completeness
174 was assessed using BUSCO v3 (Rhizobiales database, Waterhouse *et al.* 2018).

175

176 **Comparative genomics**

177 We inferred orthologs from all nine assembled genomes and reference genomes of 15
178 rhizobial strains (*Mesorhizobium*, *Ensifer*, *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*,
179 *Paraburkholderia* and *Cupriavidus*; Supporting Information Table S3), including both
180 chromosomes and plasmids, using SonicParanoid with the most sensitive parameter
181 settings (Consentino and Iwasaki 2018).

182 We extracted single-copy orthologs that were found in all nine sequenced
183 strains and two reference strains (*M. japonicus* MAFF303099 and *M. loti* TONO).

184 Orthologous groups were assigned as genes in the core genome or in the symbiosis
185 island based on the genome locations of the reference strain, *M. japonicus*

186 MAFF303099: coordinates of the symbiosis island were 4,643,427 [*intS*] – 5,255,770
187 [*trnF*-GAA], and the rest was considered to be the core genome (Kaneko *et al.* 2000).
188 We generated a multiple nucleotide sequence alignment of each single-copy orthologous
189 group using MAFFT 7.245 (Kato and Standley 2013) with the E-INS-i algorithm, and
190 extracted biallelic single nucleotide polymorphisms (SNPs) from the alignments.

191 To visualize the genetic variations among the nine sequenced strains, we
192 performed principal component analyses (PCA) based on the following datasets: 1)
193 presence/absence of orthologs, 2) the copy number variation of orthologs, 3) SNPs in
194 the core genome and 4) SNPs in the symbiosis island. PCA was performed using the
195 *prcomp* function implemented in R 3.6.1 (R Core Team (2019); [http://www.R-](http://www.R-project.org/)
196 [project.org/](http://www.R-project.org/)).

197 To characterize the genome-wide pattern of polymorphisms in these nine
198 sequenced strains, we calculated nucleotide diversity (π values for synonymous and
199 nonsynonymous sites) and Tajima's *D* for each gene using MEGA-CC (Kumar *et al.*
200 2012; Tamura *et al.* 2011).

201 To investigate the phylogenetic relationships of *L. japonicus*-associated
202 symbionts with other nodule bacteria, phylogenetic trees of each single-copy gene were
203 reconstructed using the maximum likelihood method with the program RAXML-NG
204 (Kozlov *et al.* 2019), together with orthologs from reference sequences. Prior to the tree
205 reconstruction, haplotypes were determined for each gene based on nucleotide
206 substitutions and indels. The nucleotide substitution models were selected by the Akaike
207 Information Criterion, as implemented in ModelTest-NG (Darriba *et al.* 2019). The
208 single most likely tree out of 10 search replicates was saved for phylogenetic analyses.
209 The outgroups of each phylogenetic tree were determined using the Graph Splitting

210 method (Matsui and Iwasaki 2019) based on protein sequences of each gene, under the
211 assumption that an outgroup is the most distant operational taxonomic units from the
212 focal group containing *L. japonicus*-associated symbionts. The sensitivity was set to 2.0
213 in MMseqs2 (Steinegger and Söding 2017), used in the GraphSplitting method.

214 We then characterized the topologies of maximum likelihood trees of each
215 single-copy gene based on the following criteria: 1) whether *L. japonicus*-associated
216 symbionts (nine sequenced strains in this study, *M. japonicum* MAFF303099 and *M. loti*
217 TONO) formed a single clade; 2) whether *Lotus*-associated symbionts (*L. japonicus*-
218 associated symbionts, *M. loti* NZP2037 and *M. japonicum* R7A) formed a single clade;
219 and 3) whether *Mesorhizobium* strains (nine sequenced strains in this study and other
220 *Mesorhizobium* strains) formed a single clade. This analysis was performed using in-
221 house scripts written in Python 3.

222 We also performed synteny analysis using the progressiveMauve 2.4.0 program
223 (Darling *et al.* 2010) to identify any structural variations in the symbiosis islands. The
224 symbiosis islands of each genome were first identified by aligning them with that of *M.*
225 *japonicum* MAFF303099. We then excised the region and aligned the direction using
226 SnapGene software (GSL Biotech; <https://www.snapgene.com>).

227

228 **Cross-inoculation experiments**

229 To quantify the effects of rhizobial symbionts, host plants and their interactions on plant
230 phenotypes, we performed a cross-inoculation experiment using nine sequenced
231 rhizobial strains (Supporting Information Table S1) and 15 *L. japonicus* natural
232 accessions (Supporting Information Table S2), resulting in 135 combinations in total.
233 Seeds of *L. japonicus* accessions were obtained from the Natural BioResource Project.

234 Prior to inoculation experiments, we prepared inoculant strains in the
235 logarithmic growth phase. We cultured rhizobial strains on a TY agar plate for 4 days at
236 28°C, and then picked single colonies and precultured them with shaking in 2 mL TY
237 liquid media for 3 days at 28°C. Aliquots (200 µL) of precultured strains were
238 transferred into 50 mL lots of TY liquid medium and cultured with shaking at 28°C for
239 48 h. The cultured strains were precipitated by centrifugation at 5800 g for 3 min,
240 washed with sterilized water three times and adjusted to 1.0×10^7 cells/mL (based on
241 optical density at 600 nm).

242 Partly scrubbed *L. japonicus* seeds were surface sterilized by immersion in 2%
243 sodium hypochlorite for 3 min and rinsed three times with sterile distilled water. After
244 overnight imbibition, the swollen seeds were sown onto 0.8% agar plates, incubated in
245 the dark for 3 days at 20°C and then grown at 20°C under 16/8 light/dark conditions for
246 24 h. The rooting plants were transplanted into Leonard jars (Leonard 1943) filled with
247 300 mL sterilized vermiculite with 300 mL sterilized nitrogen-free B&D medium
248 (Broughton and Dilworth 1971) and grown at 20°C under the same lighting conditions
249 for 3 days. Finally, we inoculated 20 mL of each concentration-adjusted rhizobial strain
250 into Leonard jars and grew them at 20°C under the same lighting conditions for 21 days.
251 We then harvested whole plant bodies, imaged all individuals with a high resolution
252 scanner and separated them into shoots and roots. The shoots were dried over 48 h at
253 65°C, and then the dry weights were measured. For root phenotypes, we measured the
254 numbers and areas of nodules from the scanned data. Shoot dry weight (SDW in g),
255 number of nodules (NON), total size of nodule (NOA in mm²) and nodule size per
256 nodule (NOA/NON ratio defined as NOAi in mm²) were obtained from all individuals
257 used in the experiment. We repeated all inoculation experiments twice. When we grew

258 *L. japonicus* without inoculation, plants did not form any nodules and lost most of the
259 leaves (data not shown).

260

261 **Data analysis of the inoculation experiments**

262 We performed analysis of variance to test whether genotypes of inoculant symbiont,
263 those of host plants and their interactions ($G \times G$) significantly influenced the four
264 measured phenotypes (SDW, NON, NOA and NOAi). We considered mean values of
265 each rhizobial strain (means of phenotypes of 15 plant accessions) as the rhizobial
266 quality. To quantify $G \times G$ interactions, we generated Euclidean distance matrices based
267 on the phenotypic differences between rhizobial strains by using the standardized
268 phenotypic values whose mean values of each rhizobial symbiont were set to 0, thereby
269 controlling for bacterial genetic effects. We used mean phenotype values and Euclidean
270 distance matrices for the following association analyses with the bacterial genome
271 sequences. R v. 3.6.1 was used for analysis of variance (R Core Team 2019), and the
272 Euclidean distance was calculated by using SciPy (Virtanen *et al.* 2019).

273 To determine the genomic regions associated with rhizobial quality, we first
274 performed an SNP-based association analysis that examined the correlation between
275 genome-wide SNPs and rhizobial quality using a linear model implemented by LIMIX
276 (Lipert *et al.* 2014). We then performed Mantel tests to evaluate whether the genetic
277 distance between rhizobial strains was correlated positively with the difference in the
278 variations of rhizobial quality or those of $G \times G$ interactions. We performed these
279 analyses for the core genome and symbiosis islands separately to examine which
280 genomic regions were more strongly associated with variations in rhizobial quality. For
281 the genetic distance, kinship matrices were calculated using mixmogam (Segura *et al.*

282 2012). Mantel tests were conducted using Spearman's rank correlation implemented in
283 the scikit-bio v. 0.5.4 (<http://scikit-bio.org/>) library of Python.

284 **RESULTS**

285 **Bacterial genome sequencing**

286 Nine rhizobial genome sequences were obtained using ONT MinION and Illumina
287 HiSeq sequencing. From MinION sequencing, 1,011,067 reads (mean length 6748 bp)
288 were obtained, covering a total of 6.823 Gb. After pre-processing the MinION
289 sequences, 822,488 reads were allocated into the nine samples, which ranged from
290 47,958 to 142,669 in read number and 370–1181 Mb in total length (Supporting
291 Information Table S4). From HiSeq sequencing, 32,511,368 pre-processing reads were
292 obtained and allocated into the nine samples, which ranged from 2,432,754 to 4,476,543
293 in read number and 364–671 Mb in total length. All quality-filtered reads from both the
294 MinION and HiSeq sequences were used for the *de novo* assembly analyses.

295

296 ***De novo* assembly and annotation**

297 Nearly complete assembled genomes of all nine rhizobial strains were obtained using
298 Unicycler (Wick *et al.* 2017) by combining MinION long reads and HiSeq short reads.
299 All nine rhizobial strains have large circular genomes, considered as chromosomes
300 (6.652–8.451 Mb; Fig. 1 and Supporting Information Table S1). Five of the nine strains
301 had several shorter plasmid-like circular genomes (56–654 kb), and two of the strains
302 had short fragment sequences, which were presumably contaminants (< 10 kb fragment
303 lengths and not closely related to *Mesorhizobium*). The total genome sizes excluding
304 presumable contaminant fragments ranged from 7.108 to 8.451 Mb. The genome sizes
305 of all assembled genomes were similar to that of the reference strain, *M. japonicum*
306 MAFF303099 (chromosome, 7.036 Mb; pMLa, 0.351 Mb, pMLb, 0.251 Mb; and total
307 7.596 Mb). All genomes without presumed contaminant fragments were used for the

308 following analyses. Gene predictions from all assembled genomes were conducted
309 using the RAST server (Overbeek *et al.* 2014). The number of coding sequences was
310 7173–8377, including six rRNA genes and 50–61 tRNA genes in each genome
311 (Supporting Information Table S1). The number of coding sequences was also similar to
312 that of *M. loti* MAFF303099 (7343 genes). The evaluation of completeness of gene
313 prediction by BUSCO analyses showed that all nine rhizobial strains had no missing
314 BUSCO. A few were fragmented (0.292–1.17% fragmented) and most BUSCOs were
315 complete (98.8–99.7% complete; Supporting Information Table S1). These BUSCO
316 analyses indicated that we had successfully reconstructed nearly complete genomes of
317 nine rhizobial strains.

318

319 **Ortholog analysis**

320 The newly assembled genomes, including both chromosomes and plasmids, were
321 compared with the reference genomes of 15 rhizobial strains (Supporting Information
322 Table S3). We identified a total of 15,712 orthologous groups (OGs) of genes using
323 SonicParanoid (Consentino and Iwasaki 2018). Among these OGs, 3095 were
324 conserved among 11 *L. japonicus*-associated symbionts (*M. japonicum* MAFF303099,
325 *M. loti* TONO and nine newly assembled genomes), and 3047 OGs were conserved
326 among 13 *Lotus*-associated symbionts (the same 11 *L. japonicus*-associated symbionts
327 and two *L. corniculatus*-associated symbionts, *M. japonicum* R7A and *M. loti*
328 NZP2037).

329 We obtained 2239 OGs as single-copy orthologs found in all of nine sequenced
330 genomes of symbionts and two reference strains (*M. japonicum* MAFF303099 and *M.*

331 *loti* TONO; Supporting Information Table S5). Seventy-six single-copy orthologs were
332 located in symbiosis islands based on the coordinates of *M. japonicum* MAFF303099.

333 Using all the identified OGs, we investigated the presence/absence of genes
334 present on the symbiosis islands of *M. japonicum* MAFF303099 and reported to be
335 related to symbiotic features such as nitrogen fixation, nodulation factor assembly and
336 protein secretion systems (Porter *et al.* 2019; Souza *et al.* 2012; Wang *et al.* 2014). All
337 28 nitrogen fixation-related genes (*nif*, *fdx* and *fix*) were present in the nine newly
338 assembled genomes and in other genomes of *Lotus*-associated symbionts (Fig. 2).
339 Sixteen nodulation factor assembly genes (*nod*, *nol* and *noe* genes) were present in all
340 nine genomes. All 14 type III protein secretion system (T3SS) genes were absent in one
341 *L. japonicus*-associated strain (131-3-5) and in two *L. corniculatus*-associated strains;
342 two of them (*nolB* and *nolU*) were also missing in multiple genomes of *Lotus*-associated
343 symbionts. By contrast, one *L. japonicus*-associated strain (131-3-5) had nearly the
344 complete set of the type IV protein secretion system (T4SS) genes that were absent in
345 other *L. japonicus*-associated symbionts, except for the *virB7* gene.

346

347 **Principal component analysis (PCA)**

348 PCA showed genome-wide genetic variations between strains. When based on the
349 presence/absence of orthologs, PC1 and PC2 explained 49.41% and 14.46%,
350 respectively (Supporting Information Fig. S1A), When based on copy number
351 variations, PC1 and PC2 explained 38.05% and 25.16%, respectively (Supporting
352 Information Fig. S1B). In both plots, there were two closely related pairs of strains
353 (131-2-5 and 131-3-5, L-8-3 and L-8-10) and no clear geographical clusters.

354 We extracted biallelic SNPs from each gene group (core genome 528,912 bp,
355 symbiosis island: 2497 bp) and then performed PCA separately (Fig. 3A, B). PCA based
356 on the SNPs in the core genome showed similar patterns to the one generated by
357 genome-wide ortholog profiles (Fig. 3A); there were two closely related pairs of strains,
358 which were also observed in the ortholog-based plot. By contrast, PCA using the SNPs
359 of the symbiosis island showed a distinct pattern (Fig. 3B): we again found two pairs of
360 closely related strains, but they were different from those observed in the core genome
361 or genome-wide ortholog profiles (113-3-3 and 113-3-9, 131-2-1 and 131-2-5). Overall,
362 PCA suggested that the pattern of relatedness between strains differed markedly
363 between the core genome and the symbiosis island.

364

365 **Genome-wide view of polymorphisms and gene genealogies**

366 To understand the genome-wide landscape of polymorphisms, we calculated nucleotide
367 diversity and Tajima's D statistic for each gene across the genome (Fig. 3C, D;
368 Supporting Information Table S5). Genes in the core genome showed markedly higher
369 nucleotide diversity at both synonymous and nonsynonymous sites (mean 0.4953 and
370 0.1029, respectively) compared with those of genes on the symbiosis island (mean
371 0.0250 and 0.006367, respectively). This pattern is consistent with an observation of the
372 smaller number of genes in a previous study (Bamba *et al.* 2019b), and indicates a
373 signature of horizontal gene transfer (HGT) of the symbiosis island. A steep change in
374 diversity at the borders of the symbiosis island suggests that it would have behaved as a
375 unit of HGT.

376 Tajima's D statistic of the core genome (mean 0.6990) was also markedly
377 higher than that of the symbiosis island (mean -0.5304 ; Fig. 3D; Supporting

378 Information Table S5). It is also important to note that Tajima's D statistic of the core
379 genome was generally positive, indicating the excess of alleles with intermediate
380 frequencies. This pattern would be expected if there were a clear population structure in
381 the core genome, which was indeed observed in our PCA (Fig. 3A). By contrast,
382 Tajima's D statistic of the symbiosis island was generally negative, indicating an excess
383 of rare alleles in this region, and to be expected under a scenario of recent selective
384 sweeps including HGT.

385 Patterns of phylogenetic relationships were also distinct between the core
386 genome and the symbiosis island (Fig. 3E–G, Supporting Information Table S5). In the
387 core genome, the majority of the gene trees (1555/2163 genes) showed a single
388 *Mesorhizobium* clade, whereas there was no gene tree in which *L. japonicus*- or *Lotus*-
389 associated symbionts formed a clade (Fig. 3E; Supporting Information Table S5). By
390 contrast, in the symbiosis island, 14/76 gene trees showed the *L. japonicus*-associated
391 symbionts clade, 55 genes formed the *Lotus*-associated symbionts clade and 14 genes
392 showed the *Mesorhizobium* clade (Fig. 3F; Supporting Information Table S5). These
393 results suggest that *L. japonicus*-associated symbionts mostly have the core genome of
394 *Mesorhizobium*, but their symbiosis island clearly has a different evolutionary origin,
395 supporting the HGT of the symbiosis island into the diverse genetic background of
396 *Mesorhizobium*. In addition, multiple types of topologies in the symbiosis island
397 indicated a history of recombination within the symbiosis island.

398

399 **Structural variations in the symbiosis island**

400 An alignment by progressiveMauve analysis (Darling *et al.* 2010) provided the genomic
401 regions corresponding to the symbiosis island of the reference strain, *M. japonicum*

402 MAFF303099. All of the nine newly assembled genomes harbored the symbiosis island.
403 Eight had the symbiosis island on the chromosome, but in strain 131-3-5, the symbiosis
404 island was identified on the plasmid (Fig. 1).

405 Alignment by progressiveMauve showed that the synteny of symbiosis island
406 of *L. japonicus*-associated symbionts was mostly conserved. Five conservative synteny
407 blocks were identified in the symbiosis island, and all blocks were found in eight of the
408 nine sequenced strains, except for strain 131-3-5, which lacked the whole type III
409 protein secretion system gene cluster (*nol-hrc*). This was consistent with an ortholog
410 search using SonicParanoid (Consentino and Iwasaki 2018), which revealed the lack of
411 all *nol-hrc* genes (Fig. 2).

412 The largest synteny block, 21 kb away from the start positions of symbiosis
413 islands (symbiosis islands integrase; *intS*) on *M. japonicum* MAFF303099 (Fig. 4A),
414 contained nitrogen-fixing genes (*nif*, *fix* and *fdx*) and several nodulation genes (*nod*, *noe*
415 and *nol*). We note that this largest block was inverted in the strain L-2-11. Between the
416 *nif-fdx* and *nod-nol* blocks, there was a hypervariable region containing many
417 transposase insertions (around 135 kb in *M. japonicum* MAFF303099).

418 We found that two pairs of strains harbored extremely similar symbiosis
419 islands, even including the hypervariable region (113-3-3 and 113-3-9, 131-2-1 and
420 131-2-5; Fig. 4B, C). The sequence identity values between 113-3-3 and 113-3-9 and
421 between 131-2-1 and 131-2-5 were 99.6% and 99.9%, respectively. These two pairs
422 corresponded to the pairs identified in the PCA of SNPs from the symbiosis island (Fig.
423 3B). Because their core genomes are highly different, these data also provide strong
424 evidence for recent HGT in the whole symbiosis island.

425 **Cross-inoculation experiments**

426 We performed a cross-inoculation experiment using 15 *L. japonicus* accessions and nine
427 rhizobial symbionts, resulting in a total of 135 combinations (Supporting Information
428 Tables S1 and S2). We obtained four phenotypes (SDW, NON, NOA and NOAi) from
429 1189 individuals (5–14 per combination; Fig. 5). All phenotypic traits were significantly
430 correlated with each other (Supporting Information Figs S2 and S3, all Pearson's
431 product-moment correlation $P < 2e^{-16}$). All correlation coefficients were positive (0.246
432 to 0.637), except for that between NON and NOAi (−0.351).

433 In the cross-inoculation experiment, we detected significant effects of host,
434 symbionts and their interactions on all four phenotypes (Table 1: all host, symbiont and
435 interaction effects were significant at $P < 0.001$). For all phenotypes, the effect of host
436 was the largest (partial $\eta^2 = 0.200$ – 0.268), followed by the host \times symbiont interaction
437 effect (partial $\eta^2 = 0.145$ – 0.168) and the effect of symbionts (partial $\eta^2 = 0.038$ – 0.081 ;
438 Table 1), indicating that the host phenotypes were more strongly affected by host \times
439 symbiont interactions than the sole symbiont effect. Furthermore, it is worth noting that
440 the combinations of hosts and symbionts from the same localities did not necessarily
441 show higher phenotypic values than nonnative combinations (Supporting Information
442 Table S6), which was not consistent with the pattern expected from local adaptation.

443 To understand what genes or genomic regions of rhizobial symbionts could be
444 responsible for variations in partner quality, we first quantified the rhizobial quality
445 (Supporting Information Fig. S2) and $G \times G$ effects (Supporting Information Fig. S4).
446 There were 1.233–1.413 times greater differences between minimum and maximum
447 values of variations in partner quality.

448 Genes previously reported as being involved in symbiosis would be obvious
449 candidates explaining the rhizobial variations in partner quality. Therefore, we first
450 investigated the correlations between the variations in partner quality (represented by
451 mean phenotypic values) and the presence/absence variations of these symbiosis genes.
452 Analysis of variance using the genes in which we found the presence/absence variations
453 (Fig. 2) revealed that none were significantly correlated with variations in partner
454 quality ($P > 0.05$; Supporting Information Table S7). Next, we investigated the
455 correlation between the variations in partner quality and genetic distances of genes that
456 were reported as candidate genes responsible for rhizobial quality (Klinger *et al.* 2016).
457 Mantel testing using these candidate genes found that none were significantly correlated
458 with rhizobial quality variations (Supporting Information Table S8). These results
459 suggest that known symbiosis-related genes would not be responsible for the rhizobial
460 variations in partner quality detected in the cross-inoculation experiment.

461 We then performed an SNP-based association analysis between rhizobial
462 genomes and rhizobial variations in partner quality. We found that SNPs in the core
463 genome were more strongly associated with rhizobial quality than those in the
464 symbiosis islands (Supporting Information Fig. S5). In the linear model for SDW, NON
465 and NOAi, P value distributions were strongly skewed toward small values in the core
466 genome (62.6%, 70.7% and 58.5% of SNP P values were < 0.05 , respectively;
467 Supporting Information Fig. S5A–D). These skewed P -value distributions in the core
468 genome presumably resulted from population structure and strong linkage
469 disequilibrium. On the other hand, P value distributions were not strongly skewed in the
470 symbiosis islands. Thus, the P -values were < 0.05 in 10.8% of SDW SNPs, 5.3% of

471 NON SNPs, 11.8% of NOA SNPs and 8.8% of NOAi SNPs; Supporting Information
472 Fig. S5E–H).

473 Next, we performed Mantel tests to determine whether the genetic distance
474 between rhizobial strains was positively correlated with variations in partner quality.
475 The Mantel test results between the variations of rhizobial quality for SDW and NOAi
476 were significantly correlated with the reciprocal of rhizobial core genome kinships ($P <$
477 0.05 after Bonferroni corrections; Table 2). By contrast, we did not recognize any
478 correlation between variations in rhizobial quality and the kinships of symbiosis islands.
479 These results suggest that variation in the core genome, rather than in the symbiosis
480 islands, could explain the rhizobial variations in partner quality.

481 Variations in $G \times G$ interactions were not significantly correlated with rhizobial
482 core genomes or symbiosis islands (Supporting Information Table S9). However, it is
483 noteworthy that there were variations in $G \times G$ interactions between the pairs that
484 appeared to have almost identical symbiosis islands but highly different core genomes
485 (Fig. 4B, C; 113-3-3 and 113-3-9; 131-2-1 and 131-2-5; Supporting Information Fig.
486 S4). There were no significant correlations for $G \times G$ variations between these symbiont
487 pairs, except for NOAi of 113-3-3 and 113-3-9 ($P < 0.05$ after Bonferroni corrections;
488 Supporting Information Table S10). Because the symbiosis islands are nearly identical,
489 the observed $G \times G$ variations should due to genetic variation in the core genome.

490 DISCUSSION

491 ***De novo* assembled genomes of *Lotus japonicus*-associated nodule bacteria**

492 While there have been several attempts to sequence the whole genomes of nodule
493 bacteria (Kaneko *et al.* 2000, 2002; Amadou *et al.* 2008; Lee *et al.* 2008; Reeve *et al.*
494 2010ab, 2015; Ramsay *et al.* 2013; Moulin *et al.* 2014; Wang *et al.* 2014; Shimoda *et al.*
495 2016; Nagymihály *et al.* 2017; Liang *et al.* 2018), *de novo* sequencing of multiple
496 strains associated with a single plant species has been uncommon. Here, we performed a
497 whole-genome sequencing analysis of nine *L. japonicus*-associated symbionts by
498 exploiting both long-read (Oxford Nanopore MinION) and short-read (Illumina HiSeq)
499 sequencers, which enabled us to generate high quality *de novo* assembled genomes for
500 each strain.

501 Comparative genomic analyses of these sequenced genomes provided clear
502 evidence for HGT of the symbiosis island. First, patterns of phylogenetic relationships
503 were distinct between the core genome and the symbiosis island (Fig. 3E, F; Supporting
504 Information Table S5). In the core genome, there was no gene forming an *L. japonicus*-
505 or *Lotus*-associated symbiont clade, but in the symbiosis island, 14 and 55 of 76 genes
506 showed *L. japonicus*- or *Lotus*-associated symbiont clades, respectively, indicating that
507 the evolutionary origin of the symbiosis island was clearly different from that of the
508 core genome (Fig. 3E, Supporting Information Table S5). Second, we observed a
509 marked decline in the nucleotide diversity π statistic at synonymous and
510 nonsynonymous sites and negative Tajima's *D* statistic, which are also evidence for
511 HGT (Fig. 3C, D). A steep change in diversity at the borders of the symbiosis island
512 suggests that it would have behaved as a unit of HGT, while the multiple topologies of

513 gene trees in the symbiosis island also indicate an evolutionary history of internal
514 recombination events. Third, there was a pair of strains harboring almost identical
515 sequences of the symbiosis island, but with highly distinct core genome backgrounds,
516 which is also a signature of recent HGT (Fig. 4B, C). Including our previous study of *L.*
517 *japonicus*-associated nodule bacteria (Bamba *et al.* 2019b), there have been several
518 studies demonstrating HGT of symbiosis islands based on sequences of a few genes
519 (Barcellos *et al.* 2007; Steenkamp *et al.* 2008; Menna and Hungria 2011; Koppell and
520 Parker 2012; Parker and Rousteau 2014; Lemaire *et al.* 2015; Bamba *et al.* 2016). Thus,
521 our *de novo* assembled genome data have provided multiple signatures of HGT at an
522 unprecedented level of resolution.

523 The assembled genomes also revealed blocks of conserved synteny as well as
524 extensive structural rearrangements in the symbiosis island. While Shimoda *et al.*
525 (2016) showed that there were three conserved regions (*nif*, *nod* and type III protein
526 secretion system) in the symbiosis islands of *Lotus*-associated symbionts, we found five
527 conserved blocks and many rearrangements, including an inversion and loss of genes
528 and gene clusters. As these structural rearrangements were found in the symbiosis island
529 of bacteria associated with a single legume species, rearrangements or gene gain/loss
530 could occur over a short time. We also found evidence of recombination within the
531 symbiosis island based on the topologies of gene trees (Fig. 3E, F). Signatures of gene
532 gain/loss and recombination have also been reported for several rhizobial genera
533 (*Burkholderia*, De Meyer *et al.* 2016; *Bradyrhizobium*, Sugawara *et al.* 2013; Bouznif *et*
534 *al.* 2019; Porter *et al.* 2019), and the genomic insight of the symbiosis island of *L.*
535 *japonicus*-associated symbionts is consistent with the emerging perspective that

536 recombination and gene gain/loss are not rare events in rhizobial symbiosis

537 islands/plasmids (Porter *et al.* 2019).

538

539 **Genomic regions associated with variations in partner quality**

540 By integrating the data of cross-inoculation experiments and assembled whole genomes,

541 we investigated which genomic regions could be responsible for variations in partner

542 quality. We found that plant growth was significantly influenced by host genotypes,

543 symbiont genotypes and host \times symbiont ($G \times G$) interactions (Table 1). We then

544 examined whether rhizobial variations in partner quality were explained by the

545 following genetic factors: (i) presence/absence variation of symbiosis genes, (ii) SNPs

546 in the rhizobial genomes and (iii) genetic kinships of the core genome and the symbiosis

547 island. We found that the rhizobial core genome variations explained the rhizobial

548 variations in partner quality: Mantel tests showed that the quality variations in rhizobial

549 symbionts and their core genome kinship were significantly correlated in two

550 phenotypes (SDW and NOAi; Spearman rank correlations $P < 0.05$ after Bonferroni

551 correction; Table 2), whereas the kinship of the symbiosis island was not. In the SNP-

552 based association analyses, P -value distributions of core genomes SNPs were strongly

553 skewed toward small values (Supporting Information Fig. S5), suggesting that many

554 SNPs across the genomes are correlated with variations in partner quality, consistent

555 with the results of Mantel tests. Such strongly skewed P -value distributions possibly

556 arise from extensive genome-wide linkage disequilibrium. Bacterial genomes generally

557 show strong linkage disequilibrium given their asexual reproduction (Chen *et al.* 2015),

558 which could make it difficult to pinpoint the responsible regions/genes on the genome

559 using association-based analysis.

560 Our finding of significant correlations between core genome variations and the
561 rhizobial variations in partner quality might partly explain why such variations persist in
562 legume–rhizobia mutualisms. In *L. japonicus*-associated symbionts, massive genetic
563 variations in the core genome would be maintained by recurrent HGT of the symbiosis
564 islands into diverse *Mesorhizobium* bacterial strains (Bamba *et al.* 2019b). Local
565 *Mesorhizobium* communities could thus serve as a source of standing genetic variation
566 of core genomes, which might prevent variations in partner quality from fixing even
567 under the presence of selection favoring high-quality partners: i.e. a stabilizing
568 mechanism in mutualisms (Heath and Stinchcombe 2014). In the context of the
569 mutation–selection balance model (Van Dyken *et al.* 2011; Smith *et al.* 2014), our study
570 serves to illuminate the role of HGT among symbiosis islands that indirectly supply
571 mutations in core genomes contributing to variations in partner quality.

572 A few studies have reported that variations in the symbiosis islands explain
573 rhizobial variations in partner quality, unlike our finding in *L. japonicus*-associated
574 bacteria (Klinger *et al.* 2016; Porter *et al.* 2019). Klinger *et al.* (2016) showed that
575 variations in partner quality are explained by variations in the *nifH*, *nifA*, *fixC*, *nodB* and
576 *Rleg_4928* (*fixB*) genes. Although all these genes are present in the genomes of *L.*
577 *japonicus*-associated symbionts (Fig. 2), genetic distances of these genes among strains
578 and variations in rhizobial quality were not significantly correlated, suggesting that
579 these genes do not explain the variations in partner quality in our system (Supporting
580 Information Table S8). Porter *et al.* (2019) showed that the absence of rhizobial
581 symbiosis genes lessens the quality of rhizobial symbionts. However, in our nine
582 sequenced rhizobial genomes, none of the symbiotic genes showing the
583 presence/absence of variations—all type III protein secretion system-related genes (Fig.

584 2)—were significantly correlated with rhizobial quality variations ($P < 0.05$ after
585 Bonferroni correction; Supporting Information Table S7). We note that type III protein
586 secretion system-related genes might not be involved in interactions between *L.*
587 *japonicus* and *Mesorhizobium* because dysfunctional mutants of such genes in *M.*
588 *japonicum* did not show phenotypic changes in terms of nodule-forming ability for *L.*
589 *japonicus* B129 (Okazaki *et al.* 2010), although their effects have been detected in other
590 *Lotus* species (Okazaki *et al.* 2010; Mercante *et al.* 2015).

591 We speculate that the contrasting findings between our study and previous ones
592 might have arisen in part from differences in sampling schemes and in the unique
593 history of *L. japonicus*-associated symbionts. Both Klinger *et al.* (2016) and Porter *et al.*
594 (2019) focused on rhizobial populations harboring similar core genomes. Porter *et al.*
595 (2019) used 38 strains possessing a similar core genome as a recombining population,
596 and Klinger *et al.* (2016) analyzed strains collected from nitrogen-enriched
597 experimental fields, and the nucleotide diversity of their core genomes was as low as
598 that of the symbiosis islands. By contrast, data compilation by Bamba *et al.* (2019b)
599 revealed that one of the notable characteristics of the *L. japonicus*-associated rhizobia in
600 Japan is the presence of highly diverse core genomes and the extremely low nucleotide
601 diversity of the symbiosis island. This possibly reflects recent and recurrent HGT of the
602 symbiosis island associated with the population expansion of *L. japonicus* into the Japan
603 archipelago over several thousand years (Bamba *et al.* 2019b). Therefore, it is possible
604 that the symbiosis islands of *L. japonicus*-associated bacteria analyzed in this study
605 have experienced genetic bottlenecks when *L. japonicus* migrated into the Japan
606 archipelago and are too homogeneous to serve as a source for variations in partner
607 quality.

608

609 **G × G interactions and local adaptation**

610 In our cross-inoculation experiment, we found that plant growth was significantly
611 influenced by host × symbiont (G × G) interactions, even more than symbiont genotypes
612 (Table 1), as is also observed in a few other symbiosis systems (Heath and Tiffin 2007;
613 Barrett *et al.* 2016). Such G × G interactions are suggested to underlie the selective
614 explanations for the persistence of variations in partner quality in mutualisms (Heath
615 and Stinchcombe, 2014), and our cross-inoculation experimental results support this
616 scenario.

617 We found complex G × G interactions even between two rhizobial pairs sharing
618 almost identical symbiosis islands (113-3-3 vs. 113-3-9; 131-2-1 vs. 131-2-5; Fig. 4B, C
619 and Supporting Information Fig. S4), strongly suggesting that such variations have
620 arisen in part from variations in rhizobial core genomes. Previous studies on G × G
621 interactions in legume–rhizobia mutualisms used relatively few genes, so it remained
622 unclear which genes/genomic regions were responsible for such interactions (Heath and
623 Tiffin 2007; Barret *et al.* 2015;). Here, we provide clear evidence supporting the
624 contribution of rhizobial core genome variations. However, we did not observe
625 statistically significant correlations between the variations in G × G interactions and
626 rhizobial genomic variations based on the Mantel test (Supporting Information Table
627 S9). While G × G interactions should have a genetic basis (Table 1), they might be
628 governed by polygenic factors that would not be detectable at our relatively small
629 experimental scale (15 rhizobia × 9 legume combinations).

630 Such G × G interactions in a spatial context have been a hotly debated issue in
631 legume–rhizobia mutualisms (Heath and Tiffin 2007; Heath 2010; Porter *et al.* 2011;

632 Heath *et al.* 2012; Ehinger *et al.* 2014; Harrison *et al.* 2017). The geographic mosaic
633 theory of coevolution states that the outcome of reciprocal selection between a
634 particular genotype of one species and a genotype of an interacting species will differ
635 among ecologically distinct locations (Thompson 1994, 1997, 2005; Forde *et al.* 2004;
636 Decaestecker *et al.* 2007; Laine *et al.* 2014;). According to this theory, if legume–
637 rhizobia mutualistic interactions coevolve locally, native rhizobial genotypes are
638 expected to outperform foreign rhizobial genotypes when associated with host
639 genotypes originating from the same locations. In our experiments, the NOA and SDW
640 data can be considered fitness proxies (Ratcliff *et al.* 2012; Younginger *et al.* 2017), but
641 the combinations of hosts and symbionts from the same localities did not show higher
642 phenotypic values in either of them (Supporting Information Table S6), which is not
643 consistent with the pattern expected from local adaptation. As is also discussed for the
644 maintenance of variations in partner quality, recurrent HGT of the symbiosis islands
645 into diverse *Mesorhizobium* core genomes might explain in part the absence of local
646 adaptations in the *L. japonicus* associated-symbionts, given that core genome variations
647 are strongly correlated with rhizobial variations in partner quality. With local and
648 recurrent HGT of symbiosis islands, core genome variations underlying variations in
649 partner quality might be prevented from fixation even under the presence of local
650 reciprocal selection between plants and rhizobia. By integrating the full-genome
651 sequencing of rhizobial strains and cross-inoculation experiments, this study has
652 demonstrated a scenario of how variations in partner quality could be maintained in the
653 presence of selection and HGT of symbiosis islands. More genetic studies from a plant
654 perspective would be valuable for the understanding of coevolution between plants and
655 rhizobia. This has now become possible for *L. japonicus*, where full-genome

656 resequenced data have become available for hundreds of natural accessions (Shah *et al.*

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662

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669

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918 **Table 1.** ANOVAs for growth and nodulation of plants in the inoculum treatments.

	d.f.	SDW ^a	NON ^b	NOA ^c	NOAi ^d
Host	14	0.244***	0.228***	0.268***	0.200***
Inoculant	8	0.093***	0.048***	0.038***	0.081***
Host x Inoculant	112	0.161***	0.168***	0.145***	0.162***

^a Shoot dry weight, ^b Number of nodules, ^c Total size of nodules, and ^d Nodule size per one nodule.

Numerators indicate degree of freedom for d.f and partial η^2 for each phenotype

An asterisk (***) indicates significance at $P < 0.001$

Table 2. Mantel test between partner quality variations and kinships of rhizobial genomes

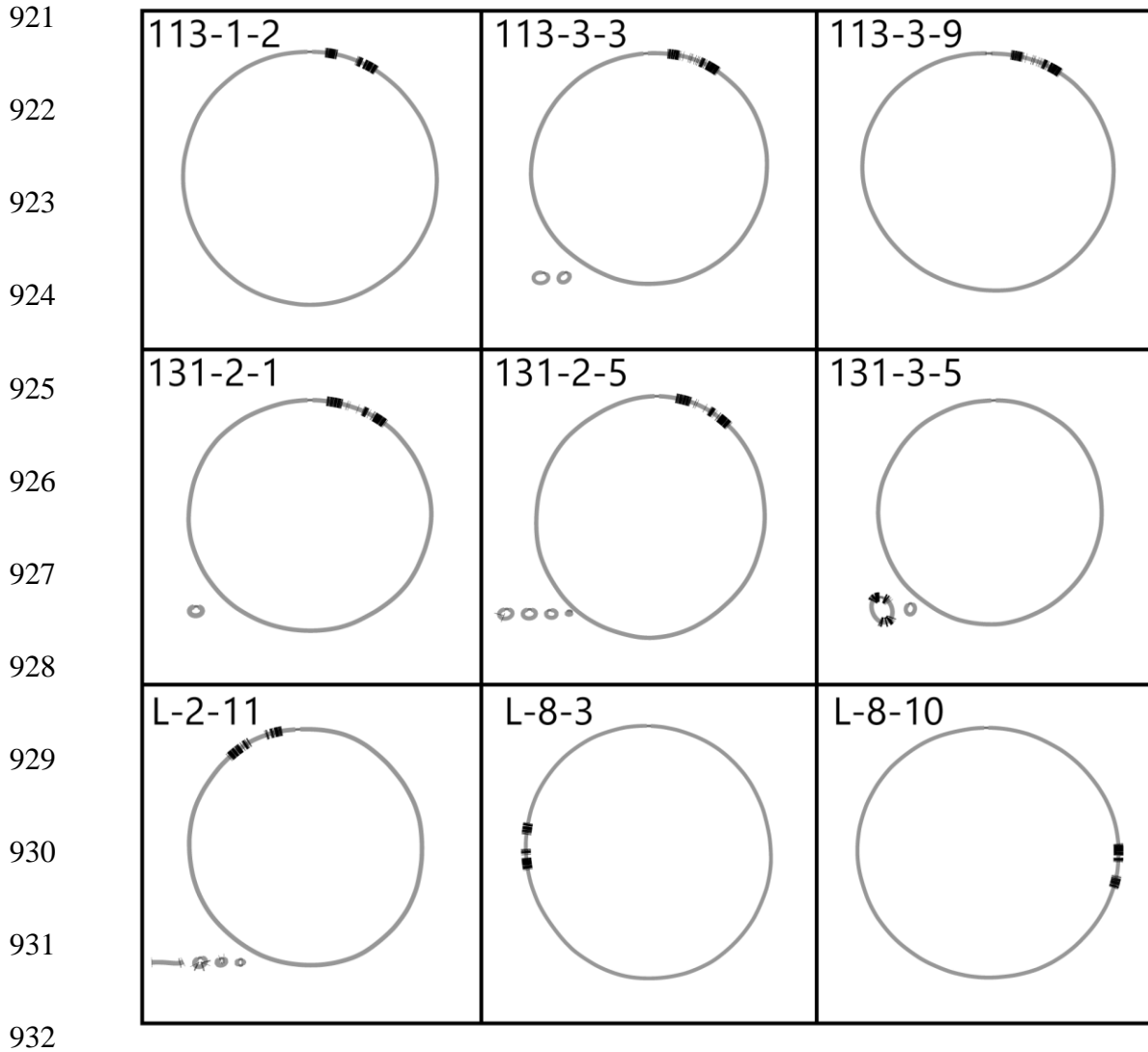
	Core ^a		Sym ^b	
	Coefficient ^a	P-value ^b	Coefficient ^a	P-value ^b
SDW	0.6561	0.0240	0.1788	1.0000
NON	0.6556	0.0560	-0.0821	1.0000
NOA	0.1030	1.0000	0.0729	1.0000
NOAi	0.9333	0.0008	0.0361	1.0000

^aNumerators indicate Spearman's rank correlation coefficient.

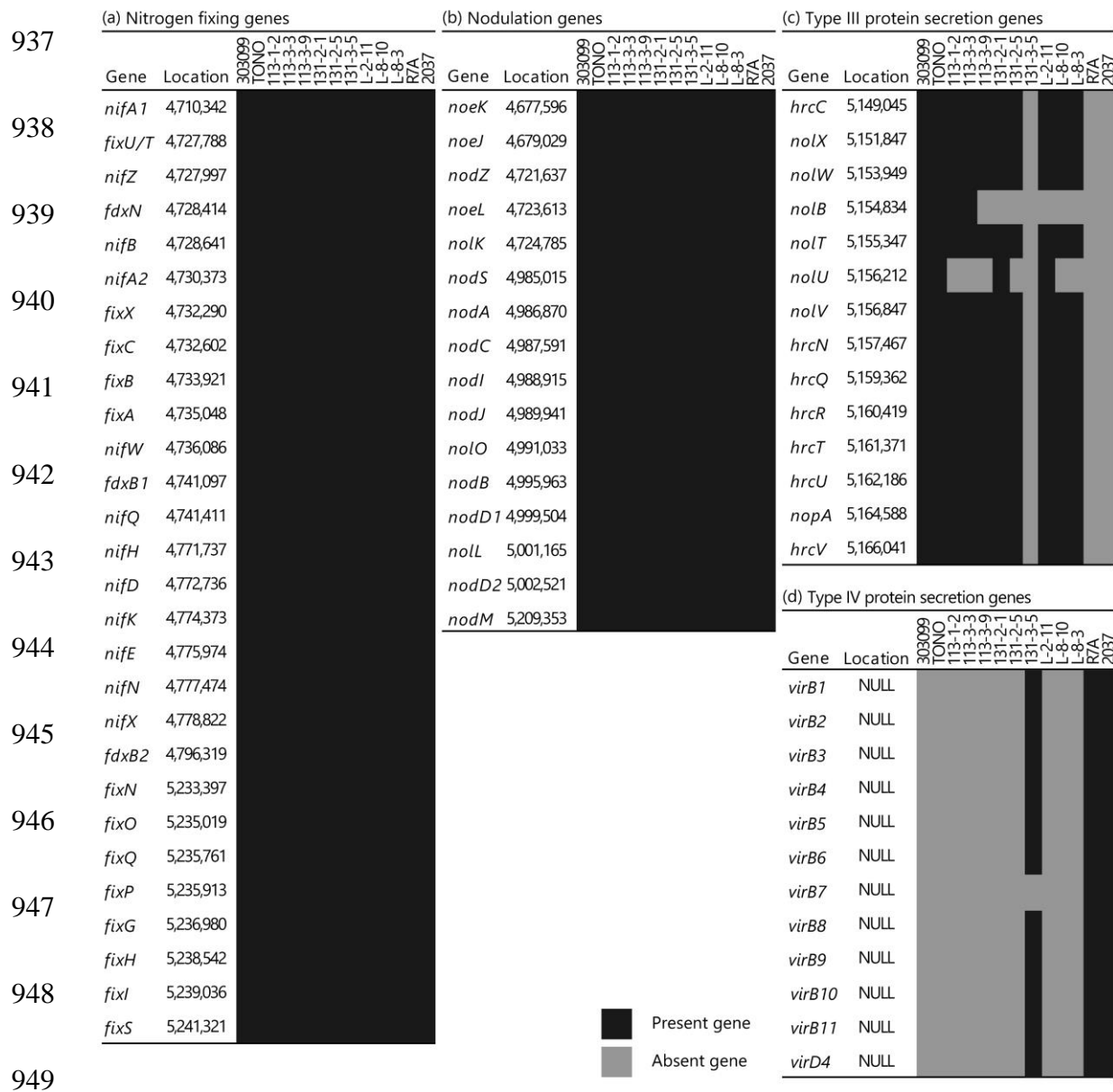
^bNumerators indicate P-value after the Bonferroni correction

919

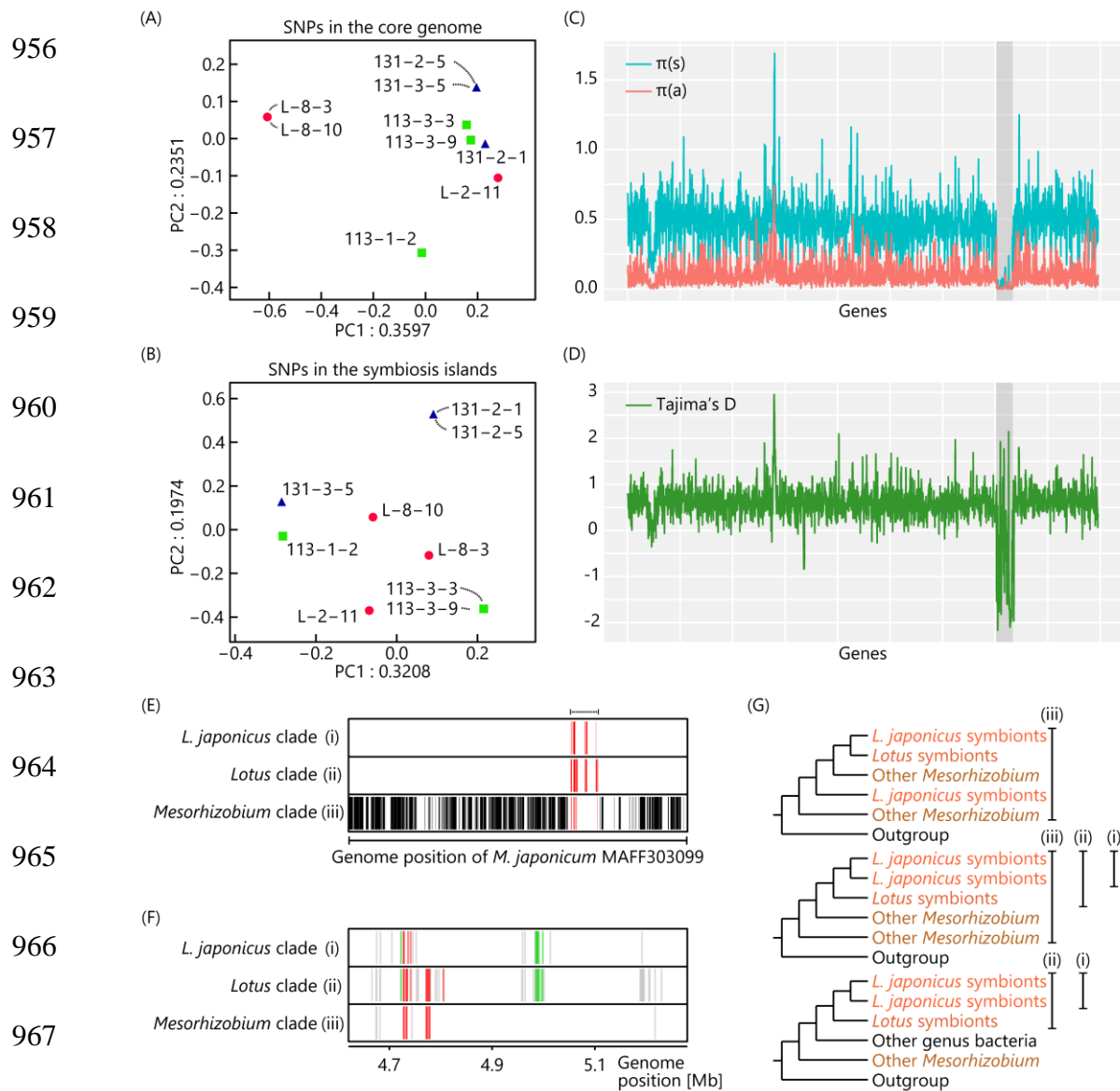
920 **FIGURES**



933 **Figure 1.** Assembly graphs of nine newly assembled rhizobial genomes. Gray circles
934 and bars indicate assembled genomes. Thick black bars on the genomes indicate genes
935 identified as homologs of genes on the symbiosis island of the reference strain, *M.*
936 *japonicum* MAFF303099.

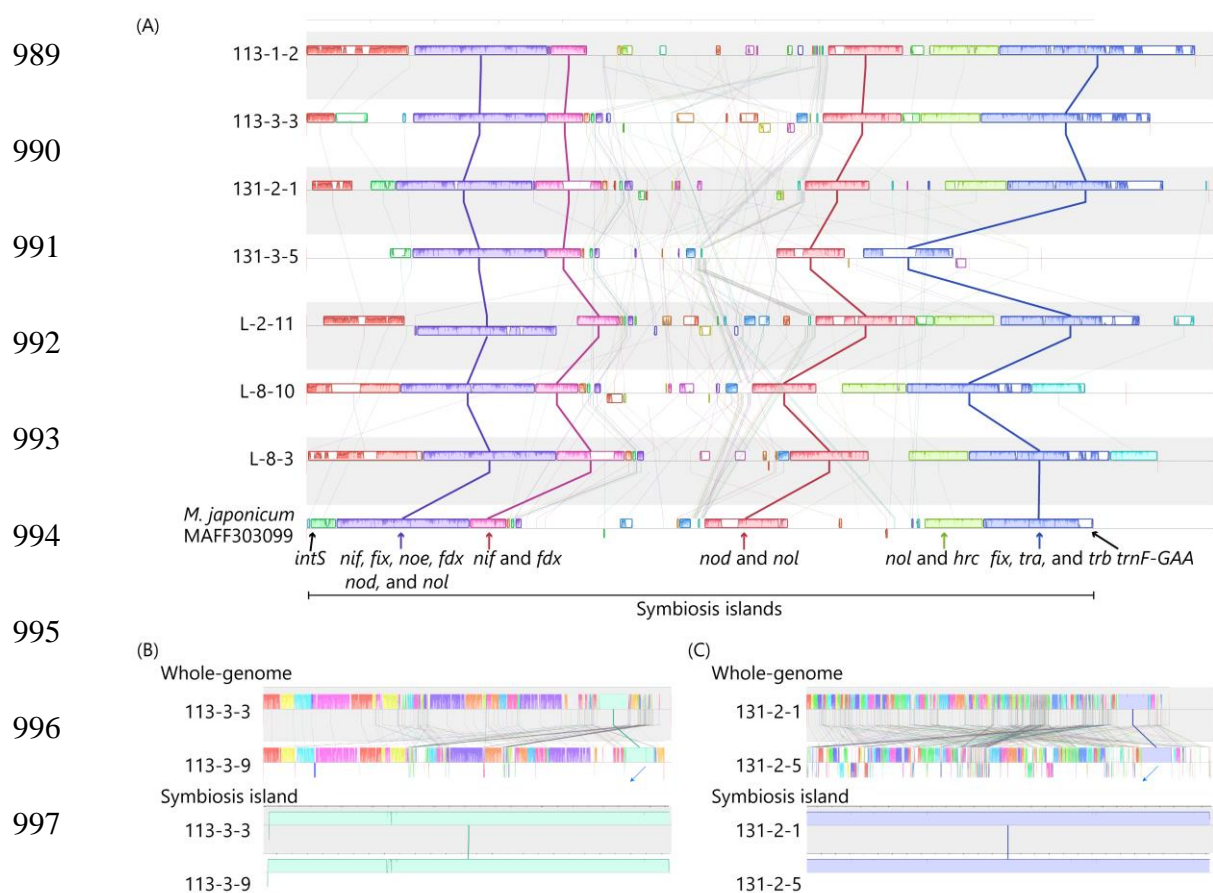


950 **Figure 2.** Presence/absence of variations among genes reported to be related to the
 951 symbiosis: (a) nitrogen-fixing genes; (b) nodulation genes; (c) type III protein secretion
 952 system genes; and (d) type IV protein secretion system genes. Each row indicates
 953 rhizobial strains with abbreviations as follows: 303099, *M. japonicum* MAFF303099;
 954 TONO, *M. loti* TONO; R7A, *M. japonicum* R7A; and 2037, *M. loti* NZP2037. Black
 955 and gray cells indicate the presence or absence of genes, respectively.

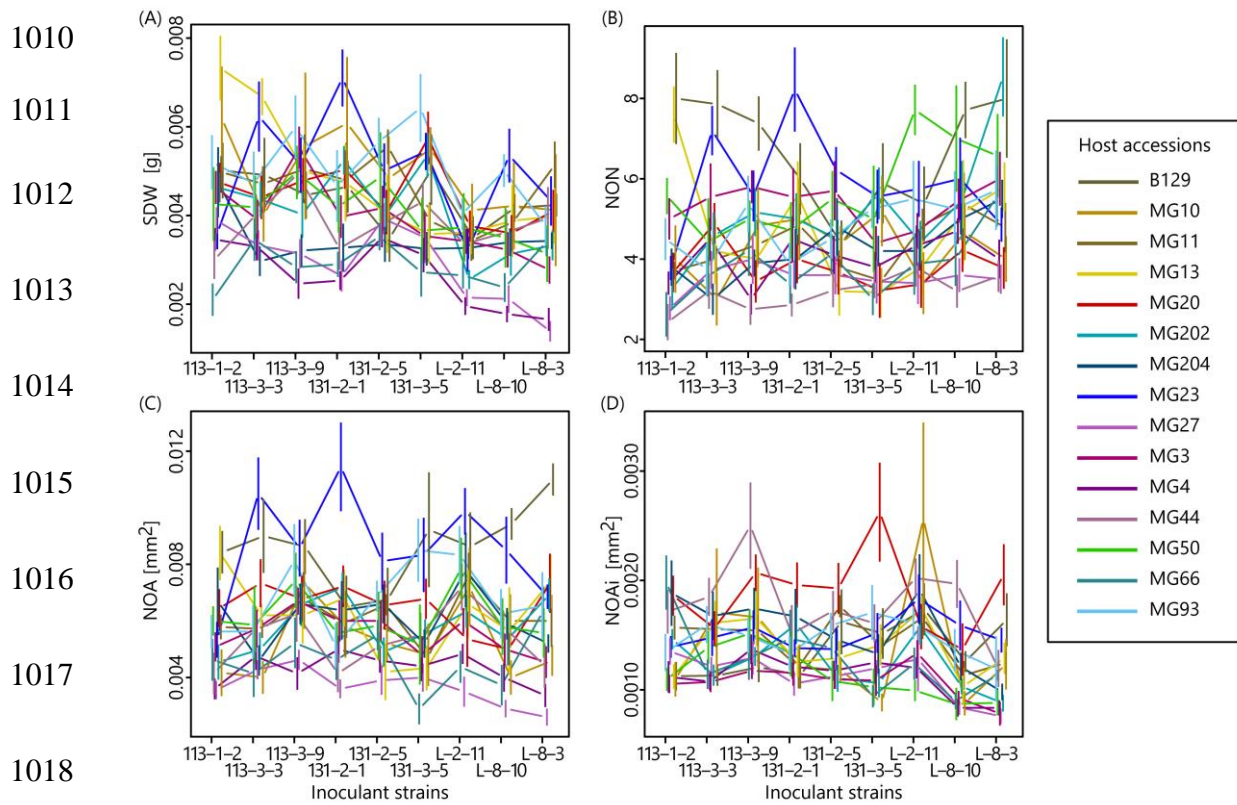


969 **Figure 3.** Genomic outlines of nine sequenced strains. (A, B) Principal component
 970 analyses of nine sequenced strains using single nucleotide polymorphisms (SNPs) in the
 971 core genome (A) and in the symbiosis island (B). Forms and colors of dots indicate
 972 sampling localities of each rhizobial symbiont with the strain type designated in
 973 brackets: blue triangle, Aomori (131); green square, Tottori (113); and red circle,
 974 Miyakojima (L). (C, D) Genome-wide distribution of nucleotide diversity statistics π
 975 (C) and Tajima's D (D), calculated for each single-copy gene described in Supporting

976 Information Table S4. The order of genes was based on the genome assembly of *M.*
977 *japonicum* MAFF303099. The shaded region indicates the symbiosis island. (C) Red
978 and blue lines indicate the nucleotide diversity statistic π at nonsynonymous and
979 synonymous sites, respectively. (E, F and G) Comparison of phylogenetic tree
980 topologies in the rhizobial whole genomes (E) and the symbiosis island (F). Bars are
981 indicated if: (i) *L. japonicus*-associated symbionts (nine sequenced strains, *M.*
982 *japonicum* MAFF303099 and *M. loti* TONO) form a single clade; (ii) *Lotus*-associated
983 symbionts (*L. japonicus*-associated symbionts and *M. loti* NZP2037, and *M. japonicum*
984 R7A) form a single clade; and (iii) *Mesorhizobium* strains (nine sequenced strains in
985 this study and other *Mesorhizobium* strains) form a single clade. (E) The black and red
986 bars indicate genes on the core genome and the symbiosis island, respectively. (F) Each
987 red, green and gray bar indicates nitrogen-fixing, nodulation and unknown genes. (G)
988 Schematic trees showing clades satisfying criteria i–iii listed above.

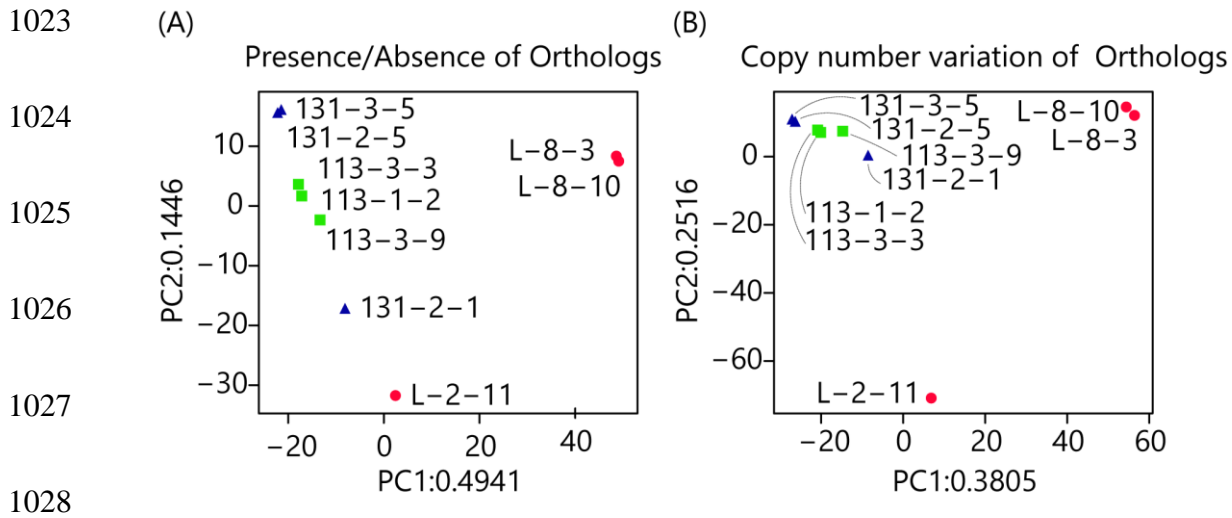


999 **Figure 4.** An alignment of the symbiosis islands of *L. japonicus*-associated symbionts,
 1000 using progressiveMauve software. Bordered and connected boxes indicate similar
 1001 sequence compositions among sequences. (A) The boxes connected by bold lines
 1002 indicate conservative genetic clusters among all *L. japonicus*-associated symbionts. The
 1003 purple and pink/purple conserved blocks, red conserved block and blue and yellow
 1004 conserved blocks are referred as *nif*-*fdx* blocks, *nod* blocks and *nol*-*hrc* blocks in the
 1005 main text, respectively. (B, C) Alignment of two pairs of rhizobial strains harboring
 1006 almost identical symbiosis islands, 113-3-3 and 113-3-9 (B) and 131-2-1 and 131-2-5
 1007 (C), generated by progressiveMauve software. The upper and lower diagrams show
 1008 alignments of whole genomes and the symbiosis islands, respectively. Boxes connected
 1009 by bold lines indicate the symbiosis islands.

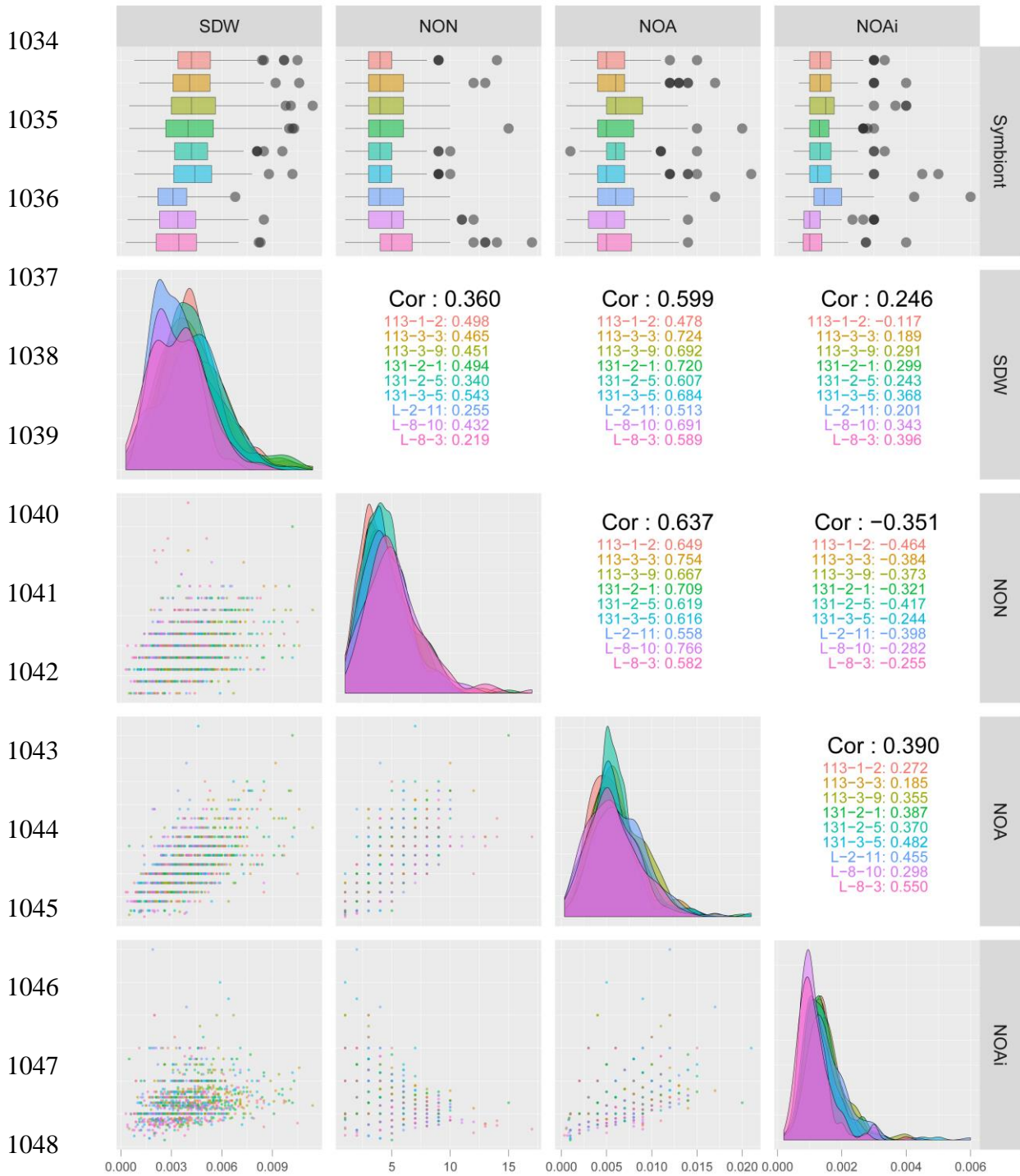


1019 **Figure 5.** Plant phenotypes of the cross-inoculation experiments, (A) shoot dry weight
1020 (SDW), (B) nodule numbers (NON), (C) total nodule size (NOA), (D) nodule size per
1021 nodule (NOAi). Mean values and standard errors (bars) are shown.

1022 **SUPPLEMENTARY FIGURES**



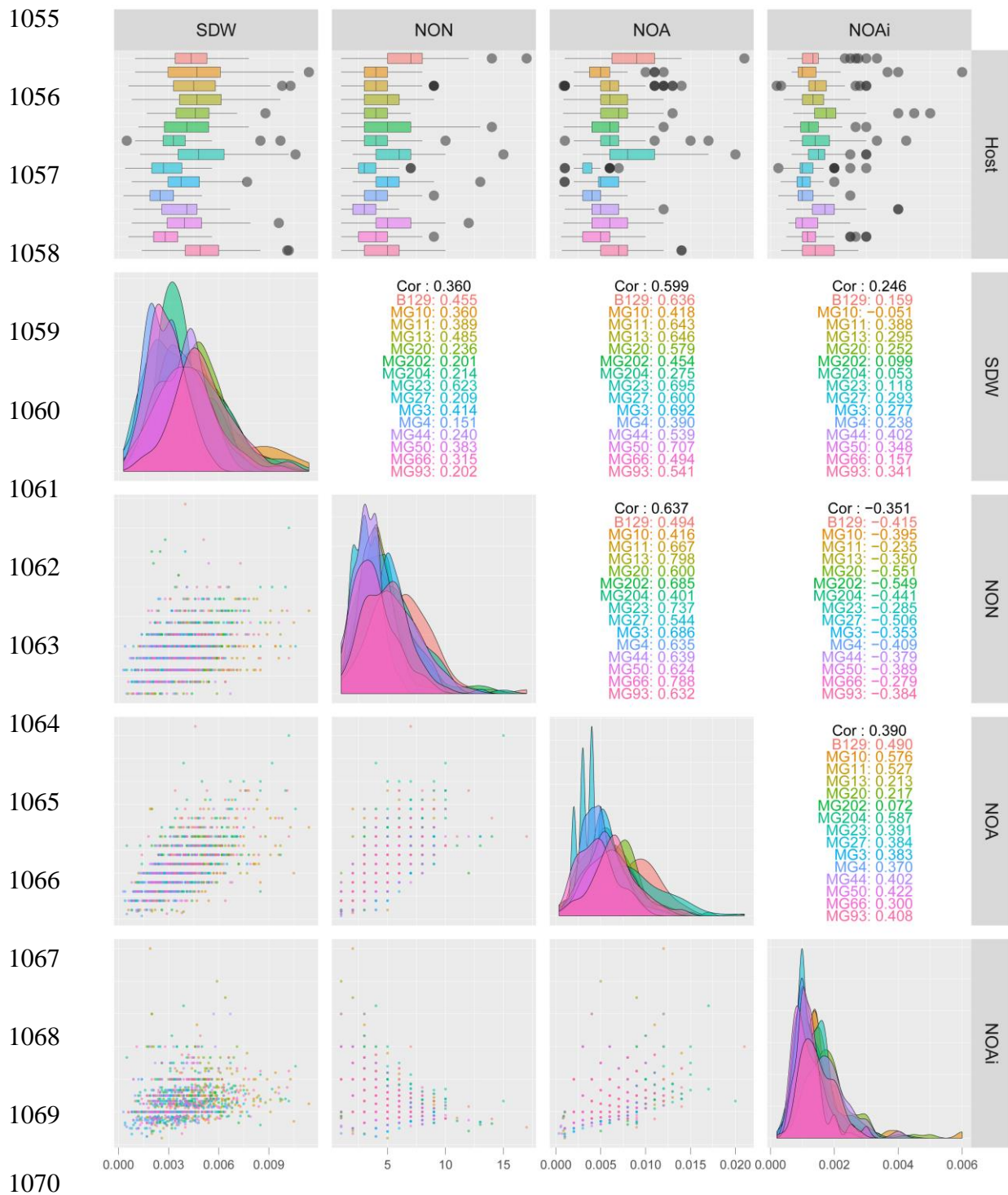
1029 **Figure S1.** Principal component analysis (PCA) results of nine sequenced strains using
1030 ortholog profiles. (A) PCA based on the presence/absence of orthologs. (B) PCA based
1031 on copy number variations. Forms and colors of dots indicate the sampling localities of
1032 each rhizobial symbiont with the strain type designated in brackets: blue triangle,
1033 Aomori (131); green square, Tottori (113); and red circle, Miyakojima (L).



1049 **Figure S2.** Correlations of phenotypes in rhizobial symbionts. Box plots show the
 1050 distribution of phenotypic values for each rhizobial symbiont, and horizontal grey bars
 1051 indicate the mean values. The x- and y-axes in each scatterplot represent phenotypic
 1052 trait values: SDW, NON, NOA and NOAi. The diagonal plots are density curves for the

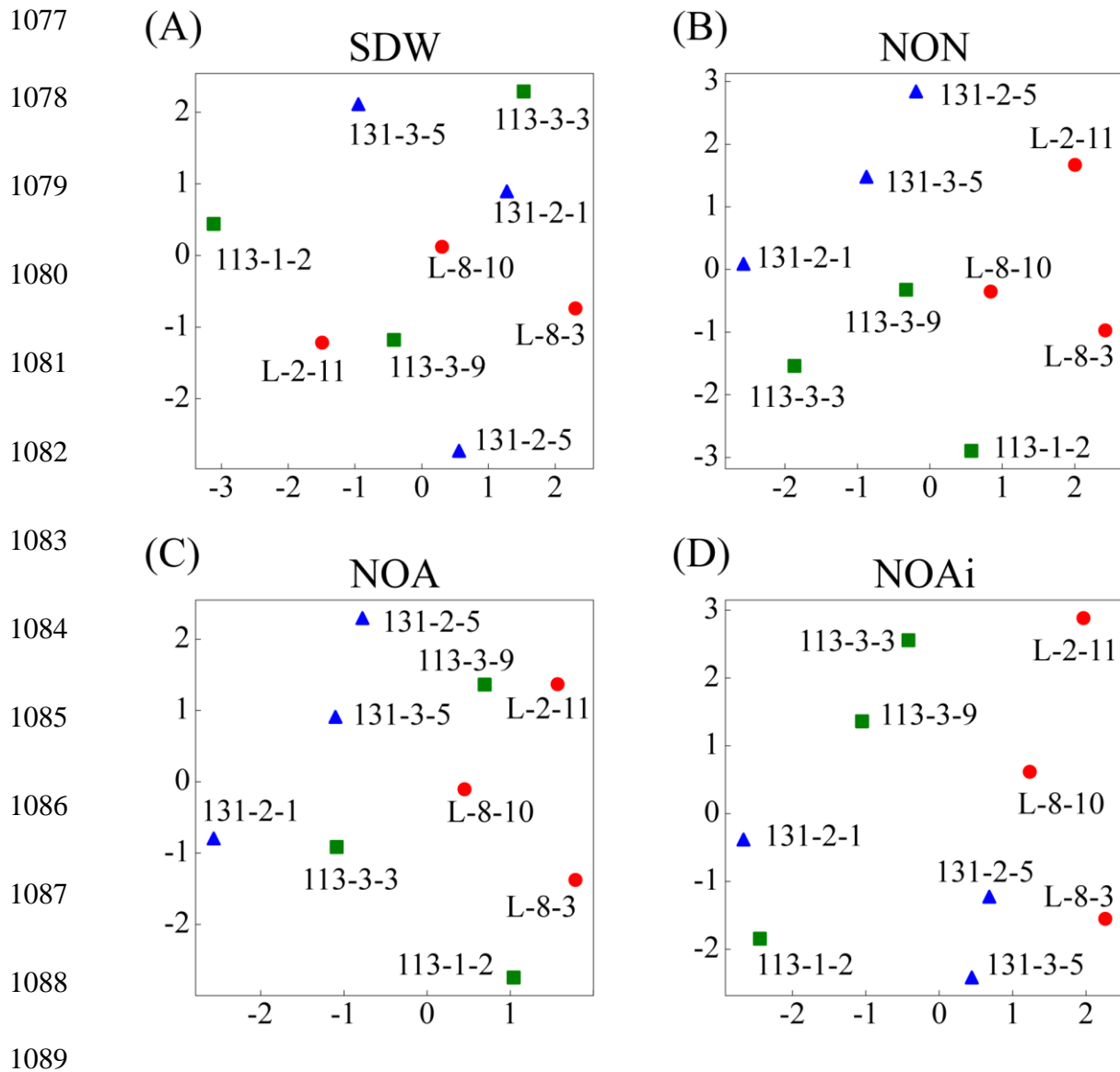
1053 individual points and Pearson's correlation coefficients are given for all phenotypes

1054 (Cor; black). Each color indicates one rhizobial symbiont.

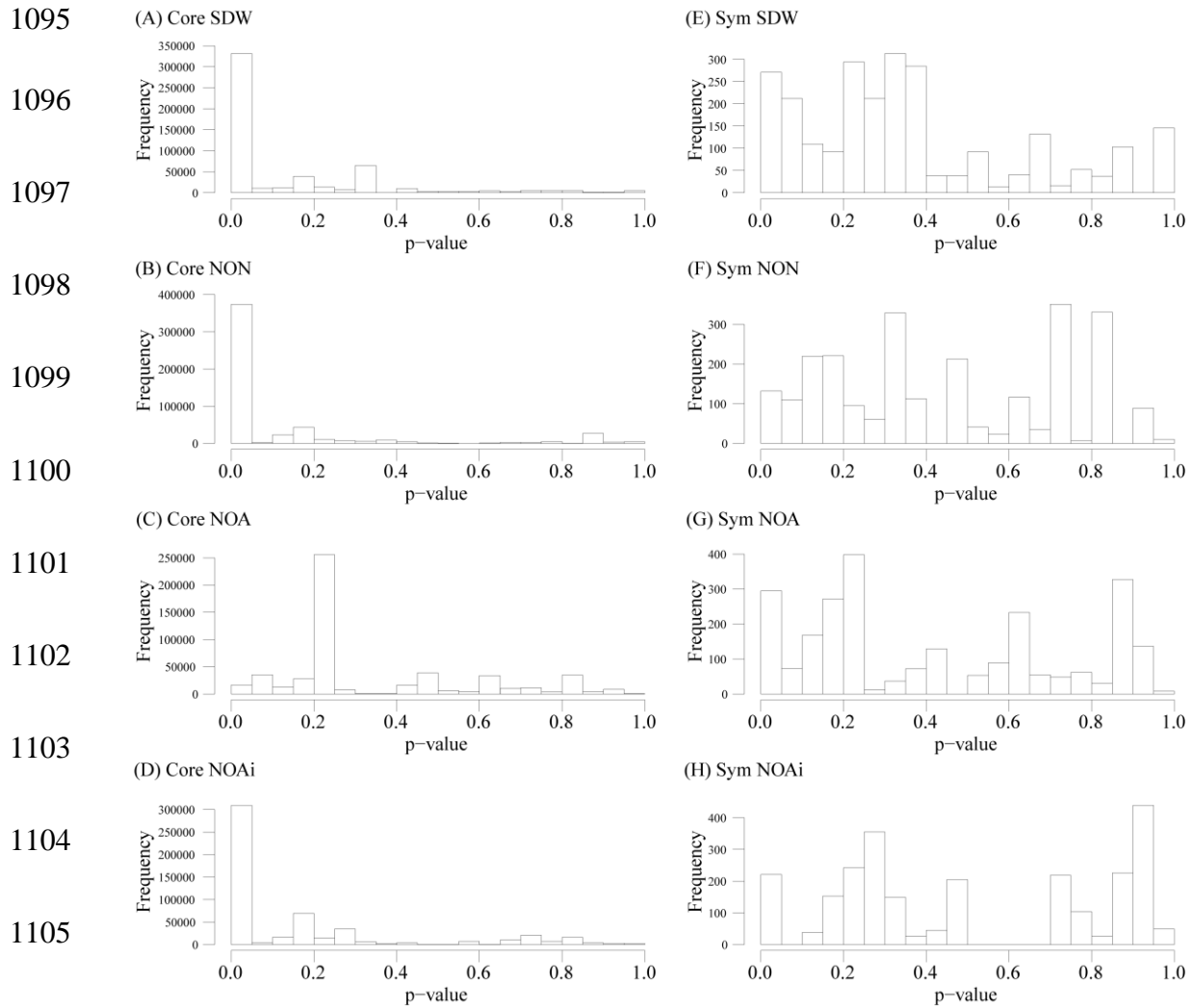


1071 **Figure S3.** Correlations of phenotypes in *L. japonicus* accessions. Box plots show the
 1072 distribution of phenotypic values for each accession, and horizontal grey bars indicate
 1073 the mean values. The x- and y-axes in each scatterplot represent phenotypic trait values:

- 1074 SDW, NON, NOA and NOAi. The diagonal plots are density curves for the individual
1075 points and Pearson's correlation coefficients are given for all phenotypes (Cor; black).
1076 Each color indicates one *L. japonicus* accession.



1090 **Figure S4.** Non-metric multidimensional scaling of variations in $G \times G$ interactions of
 1091 rhizobial symbionts: (A), SDW; (B), NON; (C), NOA; and (D), NOAi. Forms and
 1092 colors of dots indicate sampling localities of each rhizobial symbiont with the strain
 1093 type designated in brackets: blue triangle, Aomori (131); green square, Tottori (113);
 1094 and red circle: Miyakojima (L).



1107 **Figure S5.** *P*-value distributions of correlations between variations in partner quality of
1108 each phenotype and the SNPs of the core genomes (A–D) and their symbiosis islands
1109 (E–H).

1110 **SUPPLEMENTAL FILES**

1111 **Supplemental Table 1:** Summary of de novo assembly

1112 **Supplemental Table 2:** List of host accessions

1113 **Supplemental Table 3:** List of reference genomes

1114 **Supplemental Table 4:** Summary of next-generation sequencing data

1115 **Supplemental Table 5:** List of single copy orthologs

1116 **Supplemental Table 6:** Results of TukeyHSD test

1117 **Supplemental Table 7:** Results of ANOVAs 1

1118 **Supplemental Table 8:** Results of ANOVAs 2

1119 **Supplemental Table 9:** Results of Mantel test 1

1120 **Supplemental Table 10:** Results of Mantel test 2

1121