



Title	Spatial heterogeneity in genetic diversity and composition of bacterial symbionts in a single host species population
Author(s)	Kagiya, Shinnosuke; Utsumi, Shunsuke
Citation	Plant and soil, 452, 513-527 https://doi.org/10.1007/s11104-020-04583-4
Issue Date	2020-06-15
Doc URL	http://hdl.handle.net/2115/81934
Rights	This is a post-peer-review, pre-copyedit version of an article published in Plant and soil. The final authenticated version is available online at: http://dx.doi.org/10.1007/s11104-020-04583-4
Type	article (author version)
File Information	PLSO-D-19-01313_R1_without_responses.pdf



[Instructions for use](#)

1 **Spatial heterogeneity in genetic diversity and composition of bacterial**
2 **symbionts in a single host species population**

3

4 Shinnosuke Kagiya^{1*}, Shunsuke Utsumi²

5

6 ¹Graduate School of Environmental Science, Hokkaido University, North 10, West 5,
7 Sapporo, Hokkaido 060-0810, Japan (e-mail: slotapir-1035@fsc.hokudai.ac.jp)

8 ²Field Science Center of Northern Biosphere, Hokkaido University, North 9, West 9,
9 Sapporo, Hokkaido 060-0809, Japan (e-mail: utsumi@fsc.hokudai.ac.jp)

10

11

12 *Corresponding author

13 Shinnosuke Kagiya

14 Graduate School of Environmental Science, Hokkaido University, North 10, West 5,
15 Sapporo, Hokkaido 060-0810, Japan

16

17 **Abstract**

18 **Aims**

19 Revealing genetic diversity in a root nodulation symbiosis under field conditions is
20 critical to understand the formation of ecological communities of organisms associated
21 with hosts and the nitrogen cycle in natural ecosystems. However, our knowledge of
22 genetic diversity of bacterial mutualists on a local scale is still poor because of the
23 assumption that the genetic diversity of mutualistic bacteria is constrained by their hosts.

24

25 **Methods**

26 We thoroughly investigated genetic diversity of *Frankia* in a local forest stand. We
27 collected root nodules from 213 *Alnus hirsuta* seedlings covering the spatial range of the
28 continuous population, which means that *Alnus* individuals occurred in a relatively
29 homogeneous distribution in a continuous forest. Then, a phylogenetic analysis was
30 performed for the *nifD*-K IGS region, including global *Frankia* sequences from *Alnus*
31 hosts.

32

33 **Results**

34 The genetic diversity of *Frankia* detected even on a local scale measured as high as that
35 shown by previous studies conducted on a regional scale. Moreover, a genetic structure
36 analysis revealed a spatially mosaic-like distribution of genetic variation in *Frankia*
37 despite the small spatial scale.

38

39 **Conclusions**

40 The genetic diversity and composition of bacterial mutualists are heterogeneous on a local
41 scale. Our findings demonstrate that genetically different bacterial symbionts
42 simultaneously interact with a single host population and interaction partnerships
43 spatially vary. The standing variation could produce dynamic ecological and evolutionary
44 outcomes in a heterogeneous forest ecosystem.

45

46 **Key words**

47 *Alnus hirsuta*, *Frankia*, genetic diversity, local scale, *nifD-K* IGS region, nitrogen-fixing
48 bacteria, root nodule symbiosis

49

50 **Introduction**

51 Most terrestrial plants interact with microsymbionts in the rhizosphere. Root nodule
52 symbiosis between plants and nitrogen-fixing bacteria has a significant impact on the
53 nitrogen cycle in terrestrial ecosystems. In both evolutionary and applied biology, genetic
54 variation in rhizobial mutualism has attracted considerable attention (Barrett et al. 2012;
55 Miller and Sirois 1982; Robinson et al. 2000). Typically, experimental inoculation studies
56 using different strains of bacterial symbionts have reported different effects of the
57 mutualistic interactions, such as growth, nitrogen contents, and leaf size of the host plants,
58 as well as nitrogen-fixation activity of the bacteria (Dillon and Baker 1982; Hooker and
59 Wheeler 1987; Prat 1989; Sellstedt et al. 1986). In other words, it has been widely
60 acknowledged that intraspecific variation in mutualistic nitrogen-fixing bacteria greatly
61 affects host plant performance in terms of growth, survival, reproduction, and defense
62 (Ballhorn et al. 2017; Barrett et al. 2012; Dean et al. 2014; Miller and Sirois 1982; Pahua
63 et al. 2018; Prat 1989; Robinson et al. 2000), which in turn influences nitrogen-cycling
64 processes. Thus, the knowledge of genetic variation in rhizobial mutualism is essential to
65 understand not only the creation and maintenance of a symbiosis but also wider ecosystem
66 processes.

67 Nitrogen-fixing *Frankia* bacteria form nodules on the roots of actinorhizal plants.
68 Many studies have focused on legume–rhizobia symbioses due to the agricultural
69 importance, while interactions between actinorhizal plants and *Frankia* have been poorly
70 examined. Whereas, many legume plants are herbaceous, most actinorhizal plants are
71 woody (Wheeler *et al.*, 2008), and actinorhizal symbiosis is a major contributor to the
72 global nitrogen budget in forest ecosystems, playing a dominant role in forest succession,
73 especially in temperate and polar ecosystems (Kucho et al. 2010; Lawrence et al. 1967).

74 Therefore, the genetics of actinorhizal plants–*Frankia* bacteria mutualism may be more
75 important than legume–rhizobia interactions on ecosystem processes in non-agricultural
76 fields. Unraveling spatial structure of genetic diversity of mutualistic bacteria in non-
77 agricultural field is likely to be important toward an understanding of nitrogen cycling,
78 associated community dynamics, and coevolutionary dynamics in nodulation symbiosis.
79 Nevertheless, there is only a small body of literature on the genetic diversity of *Frankia*
80 in natural ecosystems (Anderson et al. 2009; Ben Tekaya et al. 2018; Benson and Hanna
81 1983; Clawson et al. 1998; Clawson et al. 1999; Huguet et al. 2001; Kennedy et al. 2010;
82 Mishra et al. 2015; Pozzi et al. 2018a; Pozzi et al. 2015; Pozzi et al. 2018b; Ridgway et
83 al. 2004; Roy et al. 2017; Simonet et al. 1994; Simonet et al. 1989; Vanden Heuvel et al.
84 2004; Wilcox and Cowan 2016).

85 Researchers recently have begun to reveal *Frankia* genetic diversity in wide
86 geographic ranges. For example, Nouioui et al. (2014) investigated the genetic structure
87 of *Frankia* on a global scale. The maximum distance of their study sites was
88 approximately 19,000 km. Kennedy et al. (2010) and Wilcox and Cowan (2016) surveyed
89 in regions where the maximum distances were 336.8 km and 165.0 km respectively. Some
90 previous studies have investigated genetic diversity of *Frankia* on small spatial scales
91 and/or from a single host species (Benson and Hanna, 1983; Clawson et al. 1999; Khan
92 et al. 2007; Mishra et al. 2015; Pokharel et al. 2011; Pozzi et al., 2015; Simonet et al.
93 1994; Simonet et al. 1989). However, the above studies assessed genetic diversity of
94 *Frankia* with small sample size per study sites. Therefore, distribution of *Frankia* genetic
95 diversity within a small spatial scale has been overlooked.

96 The most important reason why the knowledge of genetic diversity of *Frankia* is still
97 limited on a small spatial scale may be the assumption that the genetic diversity of

98 mutualistic partners is low on a local scale and in a single host species. The traditional
99 mutualistic theory has suggested that the genetic diversity of mutualistic partners is
100 constrained by hosts and could be decreased by the hosts' stabilizing mechanisms, such
101 as partner choice and sanction (Archetti et al. 2011; Heath and Stinchcombe 2014).

102 It should also be noted that most previous studies compared genetic variation in
103 *Frankia* among host plant species. This is because the focus has mainly been on the
104 symbiotic host specificity in this mutualism (i.e., differences in the infectivity of rhizobial
105 symbionts among host plant species; Baker 1987; Jiabin et al. 1985; Mirza et al. 2009).
106 In natural ecosystems, different host species commonly associate with phylogenetically
107 different *Frankia* strains (Du and Baker 1992; Normand et al. 1996). For this reason, most
108 previous studies have compared genetic variation of *Frankia* among multiple host species
109 to an understanding of coevolutionary history and effects of actinorhizal mutualism.

110 However, the knowledge of genetic diversity of *Frankia* on a small spatial scale (e.g.,
111 seed dispersal range: many seeds of *Alnus* individuals dispersed within *c.* 140 m along a
112 river (Cunnings et al. 2016)) should be required to understand outcomes of considerable
113 variation in current actinorhizal ecological interactions, such as the effectiveness of the
114 bacteria in growth and survival of the host plants. This is because effects of rhizobial
115 mutualism often depend not only on genetic variation of mutualistic bacteria but also on
116 intraspecific variation of host species (Caldwell 1966; Hayashi et al. 2012; Heath and
117 Tiffin 2007; Yamakawa et al. 2003). For example, nodulation rates of *Frankia* strains
118 could also differ among intraspecific host individuals (Hahn et al. 1988). In fact, large
119 genetic variation in a host plant population, including actinorhizal and legume species, is
120 also ubiquitous in a natural forest stand (Ager et al. 1993; Kagiya et al. 2018; King and
121 Ferris 1998; Wickneswari and Norwati 1993) Our previous study revealed large genetic

122 variation in a single *Alnus* species in a continuous natural forest (20 km × 70 km; Kagiya
123 et al., 2018). Leaf traits, such as C:N ratio, leaf mass per area (LMA), and herbivory rate,
124 varied with the genetic variation and localities with the forest. Therefore, we should pay
125 attention to genetic diversity of rhizobial bacteria and its spatial heterogeneity on a small
126 spatial scale, which may be crucial to determining outcomes of ecological interaction
127 between rhizobial bacteria and actinorhizal host plants under natural ecosystem
128 conditions.

129 In this study, our goal is to elucidate spatial structure in genetic diversity and
130 composition of mutualistic bacteria even in a local population of single host species (a
131 single-host–population scale). Specifically, we sought to answer the following questions:
132 (1) how diverse genetically is *Frankia* bacteria within and across local sites, (2) do the
133 genetic compositions of *Frankia* bacteria differ among local sites, and (3) what is the
134 spatial genetic structure of *Frankia* in a natural forest. For the purposes of the study, we
135 focused on the *A. hirsuta*–*Frankia* symbiosis in a natural forest in northern Hokkaido,
136 Japan. Actinorhizal populations in this forest region are dominated by a single *Alnus*
137 species, *A. hirsuta*. The genetic variation of *A. hirsuta* within the forest has been
138 determined by a genome-wide analysis (Kagiya et al. 2018). We continuously
139 investigated the genetic diversity of *Frankia* bacteria in the *Alnus* populations at intervals
140 of *c.* 100 m (the maximum distance between host populations is 43.476 km; 213 seedlings
141 in total).

142

143 **Materials and Methods**

144 Host species and nitrogen-fixing bacteria

145 *Alnus hirsuta* (Betulaceae; *Alnus incana* ssp. *hirsuta* Spach; Chen and Li 2004; Ren et al.
146 2010) is a deciduous broadleaf tree and an early successional species. It is widely
147 distributed in temperate riparian forests of Japan, northeastern China, Korea, and Russia.
148 *Alnus* trees have the following characteristics as foundation species in a riparian forest
149 ecosystem (Ellison et al. 2005; 2010): (1) they are a dominant species in early succession
150 forests, (2) they support diverse arthropod species (Kagiya et al. 2018; Nyeko et al. 2002),
151 and (3) they are actinorhizal species able to form partnerships with nitrogen-fixing
152 actinobacteria, *Frankia* sp. (Frankiaceae) forming nodules in their roots, which seem to
153 greatly affect ecosystem processes such as nutrient cycling. *Frankia* bacteria have the
154 ability to convert atmospheric nitrogen into ammonia, and are free-living soil microbes
155 but some are obligate symbionts (Benson and Dawson 2007).

156

157 Root nodule sampling

158 Our study sites are located in and around the Uryu Experimental Forest (44° 030–290N,
159 142° 010–200E) of Hokkaido University in northern Hokkaido, Japan. This experimental
160 forest is a continuously mixed conifer–broadleaf forest of *c.* 25000 ha. One nodule was
161 collected from each of the roots of 213 *A. hirsuta* seedlings (DBH: < 2 cm) from five
162 riparian areas of the forest (BT, DRE, DRW, SE, and UT; the maximum distance between
163 our areas was 43.5 km; Fig 1) because the host trees are mainly found along rivers, and
164 streams are considered one of the primary dispersal pathways of *Frankia* bacteria (Arveby
165 and Huss-Danell 1988; Huss-Danell et al. 1997). Seedlings from which we collected root
166 nodules were selected from 17 sites from the riparian areas. Sampling root nodules from
167 *A. hirsuta* seedlings would allow us to collect samples continuously from a whole forest
168 and to estimate genetic diversity and composition of *Frankia* which interact with a single

169 host plant population at present. Sampling was conducted from June to September 2016.
170 The distance between sampling points in each site was more than 100 m. *Alnus hirsuta* is
171 the only actinorhizal species in this forest.

172

173 Molecular Analyses

174 The collected nodules were surface-sterilized using 10% (v/v) Clorox bleach. DNA was
175 extracted from root nodules using DNeasy Blood & Tissue Kit (Qiagen). The nodules
176 were sliced using sterilized razor blades and crushed using sterilized homogenization
177 sticks. The crushed lobes were heated to 37 °C for 30 min. with a 25 µl Proteinase K.
178 Polymerase chain reaction (PCR) was performed to amplify a 496-bp fragment of a *nifD*-
179 K intergenic spacer region with the *Frankia*-specific primer pair, *nifD*1310frGC (5'-CGC
180 CAG ATG CAC TCC TGG GAC TAC T-3'), and *nifKR*331frGC (5'-CGG GCG AAG
181 TGG CTG CGG AA-3'). We focused on intragenetic variation of *Frankia* based on the
182 *nifD*-K IGS region. The genetic marker is considered to be one of the useful genetic
183 markers for resolution at the species level of *Frankia* because the genetic region includes
184 higher variable than ribosomal RNA (Anderson et al. 2009; Mishra et al. 2015). PCR
185 amplification was performed as follows: 1 cycle at 95 °C for 2 min, followed by 35 cycles
186 of 95 °C for 1 min and 64 °C for 5min, and a final step of 1 cycle at 72 °C for 5 min. All
187 successful PCR products were cleaned using an ExoSAP master mix containing 0.5 µl
188 Exonuclease I (TaKaRa), 0.5 µl Shrimp Alkaline Phosphatase (SAP; New England
189 BioLabs), and 2.0 µl sterile deionized water. Incubation using a thermal cycler was
190 conducted with ExoSAP at 37 °C for 20 min and at 80 °C for 15 min. These products
191 were sequenced with an automated sequencer (3730xl DNA Analyzer, Applied
192 Biosystems). DNA sequence chromatograms were manually checked using FinchTV

193 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; <http://www.geospiza.com>), and sequences were
194 aligned using MEGA 7.0.21 (Kumar et al. 2016). Finally, nucleotides in obtained
195 sequences were checked to remove sequences of low reliability. In total, 201 sequences
196 were used for subsequent analyses.

197 Operational taxonomic unit (OTU) separation was performed to classify the 201
198 sequences at a 97.0%-threshold, using the CD-Hit program (Li and Godzik 2006). This
199 threshold was decided based on the statistical method detailed in Pölme et al. (2014).
200 These sequence data were deposited in the DNA Data Bank of Japan (DDBJ) with
201 accession no. LC482655-LC482672. Phylogenetic trees were constructed using
202 Maximum Likelihood (ML; bootstrap analyses with 1000 replications; Fig 2) and
203 Neighbor-Joining (NJ; bootstrap analyses with 10000 replications; Fig S1) based on the
204 Kimura 2-parameter evolutionary model (Kimura, 1980) with a discrete gamma
205 distribution selected by evolutionary model selection procedure in MEGA 7.0.21 (Kumar
206 et al. 2016). The phylogeny included the *nifD*-K locus of uncultured *Frankia* bacteria
207 obtained from two *Alnus* species, *A. incana* (ssp. *tenuifolia* (Anderson et al. 2009) and
208 ssp. *rubra*) and *A. viridis* (Anderson et al. 2009), and nine varieties of *Myrica rubra* (var.
209 *biji*, var. *baimei*, var. *dongkui*, var. *muye*, var. *shuimei*, var. *wandao*, var. *wumei*, var.
210 *yangliu*, var. *zaoda*; He et al. 2004), as well as a cultured ACN14a strain, whose host is
211 *A. viridis* (Normand et al. 2007). Other *Frankia* sequences from different actinorhizal
212 species, *Hippöphae salicifolia* (Mishra et al. 2015), three *Coriaria* species (*C. myrtifolia*,
213 *C. japonica*, *C. arborea*; Nouioui et al. 2014), *Elaeagnus angustifolia*, *Datisca glomerata*,
214 and *Casuarina equisetifolia* (Normand et al. 2007), were also included as outgroups.
215 These sequences covered *Frankia nifD*-K sequences of almost actinorhizal hosts in
216 GenBank. These sequences were obtained from GenBank. All positions with less than

217 95% site coverage were eliminated. Both ML and NJ phylogenetic trees were generated
218 with MEGA 7.0.21 (Kumar et al. 2016). To describe relationships between *Frankia* OTUs
219 and areas, we also generated the ML phylogeny with 1000 bootstraps, excluding the
220 sequences obtained from the database (Fig 4).

221 To analyze the spatial genetic structure of *Frankia* in a natural habitat, analysis of
222 molecular variance (AMOVA) was performed with 9999 permutations, using GenAlex
223 6.5 (Peakall and Smouse 2012). The five riparian areas were divided into 2–5 sites each
224 to analyze the genetic structure within the areas.

225

226 Analysis of *Frankia* diversity

227 To estimate spatial heterogeneity of the *Frankia* composition on a single-host–population
228 scale, Bray-Curtis dissimilarity index was calculated. The total number of each *Frankia*
229 OTU was used for the data set at the site- and area-level. The data set was standardized
230 to unified scale [0; 1], dividing by total number of each site/area, because sample sizes
231 were different among sites/areas. The significance of *Frankia* composition dissimilarity
232 among areas was analyzed using permutation MANOVA (PERMANOVA) with 9,999
233 permutations. To visually summarize the dissimilarity among sites, non-metric
234 multidimensional scaling (NMDS) in a two-dimensional space was performed. All
235 calculations in above were performed using the R package vegan 2.5-6 (Oksanen et al.
236 2019) with the R 3.6.1 software.

237 To consider spatial autocorrelation in the *Frankia* OTU compositions, we
238 calculated spatial distance among sites and areas. Spatial distance was calculated based
239 on location of each site/area with Great Circle distance method, using the R package sp
240 1.3-1(Pebesma et al. 2018). The location was calculated centroid location as the averaged

241 latitude and longitude of each *A. hirsuta* seedling. Spatial autocorrelation of *Frankia*
242 compositions was analyzed by Mantel tests with 9,999 permutations. These tests were
243 performed using the vegan package.

244

245 **Results**

246 Genetic diversity of *Frankia* on a single-host–population scale

247 To classify *Frankia* in the study forest, we used OTU methods based on the genetic
248 similarity of the *nifD*-K loci. A total of 18 OTUs were obtained at a 97.0% similarity
249 based on the *nifD*-K loci of *Frankia* in the forest (Table S1, Fig 2). This 97.0% threshold
250 for OTUs is likely to be relevant to represent the phylogenetic relationship of *Frankia*
251 strains based on the *nifD*-K ITS region because sequences of all samples were clearly
252 clustered to each clade of single OTU (Fig S2). The phylogenetic trees indicated that
253 obtained OTUs widely spread in the almost range of *Alnus*-infective clade (Fig 2, S1).
254 Each of the three most common OTUs, OTU01, OTU02, and OTU03, was placed into
255 phylogenetically different clades (Fig 2, S1). Thus, our result demonstrated that
256 genetically diversified strains co-occurred in the forest. In addition, both OTU06 and
257 OTU07 were genetically close to OTU02, and both OTU04 and OTU05 were genetically
258 close to OTU03. Some *Frankia* sequences in this study were phylogenetically close to
259 bacteria from *A. incana* ssp. *tenuifolia* or *A. viridis*, which were belonging to different
260 clades between the host species (Anderson et al. 2009; 2013). Additionally, noted that
261 seven of these OTUs (OTU01–OTU07) were obtained from multiple samples, while the
262 rest (OTU08-OTU18) was rare singleton (Fig 4, S2).

263 To illustrate differences in *Frankia* OTU diversity (i.e., the total number of OTUs
264 in each site) among the sites with the standardization of sample size, we generated a

265 rarefaction curve for each site (Fig 3). OTU diversity was greater in BT, DRE, and DRW
266 areas than in SE and UT areas. While the rarefaction curves of SE and UT areas show the
267 saturation of the total OTU number, those of BT, DRE and DRW areas indicated there
268 was likely to be still undetected OTUs in each area.

269

270 Differences in *Frankia* composition

271 The three most abundant OTUs (i.e., OTU01, OTU02, and OTU03) were commonly
272 found throughout the entire sampling areas (Fig 4), indicating a sympatric coexistence of
273 these haplotypes. However, other OTUs were localized to parts of different sampling
274 areas. OTU04, OTU05, OTU06, and OTU07 were detected in seedlings from three
275 riparian areas (BT, DRW, and DRE). These results indicated the spatial heterogeneity in
276 *Frankia* compositions within a single-host–population scale.

277 This finding was supported by NMDS community ordination, in which *Frankia*
278 compositions were diversified among areas within a single-host–population scale (Fig 5).
279 The stress of the NMDS was 0.070, indicating a good representation of the data in two-
280 dimensional ordination plot. The significant differences in *Frankia* OTU compositions
281 were detected (PERMANOVA; $P < 0.05$).

282

283 Spatial genetic structure of *Frankia*

284 The AMOVA indicated a significant genetic differentiation in *Frankia* genetic
285 communities among riparian areas and sites (Table 1). In addition, no significant
286 correlations were detected between the dissimilarity of *Frankia* compositions and spatial
287 distance (at site level: $r = -0.1037$, $P = 0.6967$; at area level: $r = 0.4897$, $P = 0.1833$). This

288 suggested that spatial structure of *Frankia* compositions was unlikely to be resulted from
289 spatial autocorrelation.

290 Overall, the spatial heterogeneity in *Frankia* genetic variation was due to the
291 differences in both OTU diversity and composition of *Frankia* strains.

292

293 **Discussion**

294 This study clearly demonstrated that multiple *Frankia* genotypes coexist even in an area
295 within a single-host–population scale. *Frankia* genetic diversity in a single-host–
296 population scale is comparable with previous studies that analyzed the *nifD*-K genetic
297 region of *Alnus*-infection *Frankia* from multiple hosts and/or in regional scales.
298 Rarefaction analysis also showed that the existence of undetected OTUs is also expected
299 in some sites. Furthermore, differences in *Frankia* genetic diversity and composition were
300 detected even within the small spatial scale (Fig 4, 5). These results suggest that
301 actinorhizal host individuals within the population can interact with different *Frankia*
302 genotypes.

303

304 *Frankia* diversity and composition on small spatial scales

305 The maintenance mechanisms of sympatric coexistence of phylogenetically distant
306 *Frankia* strains can contribute to understand the spatial heterogeneity in *Frankia* diversity
307 and composition on local scales. Three factors can explain why various genotypes of
308 mutualistic partners coexist in the same habitat (Heath and Stinchcombe 2014): (1) a
309 different selection on each genotype of the partners by genetic variation in hosts (i.e., G

310 $\times G$: genotype-by-genotype interactions), (2) genetic trade-offs between bacterial strains,
311 and (3) different functions of multiple *Frankia* genotypes.

312 First, in natural ecosystems, it is likely that the genetic structure of *Frankia* is greatly
313 restricted by the hosts' phylogenetics (Anderson et al. 2009; Pöhlme et al. 2014; Pozzi et
314 al. 2018). Mutualistic benefits for host plants from root-nodulating symbionts also differ
315 among different genotypes within a host species, as well as exhibiting interspecific
316 variation (Caldwell 1966; Hayashi et al. 2012; Heath and Tiffin 2007; Yamakawa et al.
317 2003). Therefore, the sympatric coexistence of phylogenetically distant *Frankia* strains
318 on a single-host-population scale may be at least partially explained by intraspecific
319 variation of the host plant *A. hirsuta*. Determining the effects of intraspecific variation in
320 a host species with $G \times G$ interactions in mutualism may be important to understanding
321 how a stable coexistence of genetically diverse mutualistic partners is sustained on a small
322 spatial scale.

323 Second, genetic trade-offs between different mutualism-related traits, if existent, can
324 contribute to the maintenance of a stable coexistence of different *Frankia* strains. For
325 example, if mutualistic efficiency is driven by a trade-off with the ability to compete,
326 mutually efficient *Frankia* strains can sympatrically coexist with inefficient
327 *Frankia* strains that have an advantage in intrageneric competition (Ferriere et al. 2002;
328 Hoeksema and Kummel 2003). In actinorhizal symbiosis, *Alnus* trees often interact with
329 different phenotypes of *Frankia* bacteria, including spore-positive strains hosting
330 abundant sporangia inside plant cells, and sporangia-free, spore-negative strains (Pozzi et
331 al., 2015; Torrey, 1987). Infectivity and nitrogen-fixing activity might be negatively
332 associated between the spore-positive/negative strains (Markham, 2008; Pozzi et al.,
333 2015).

334 Third, different functions of *Frankia* genotypes may contribute to the maintenance of
335 their genetic variation within the same location. Nitrogen resources from rhizobial
336 symbionts increases not only the growth of the host plants, but also their resistance to
337 herbivores (Ballhorn et al. 2017; Dean et al. 2014; Thamer et al. 2011). In addition,
338 nitrogen fixed by associated rhizobacteria, including *Frankia*, can be stored in nodules
339 and transported to aerial parts as these specific forms, such as amides and ureides (Berry
340 et al. 2011). If the genetic variation of *Frankia* strains is responsible not only for the
341 nitrogen supply but also the different forms of nitrogen, multiple functions of genetically
342 diverse *Frankia* may complementally improve the overall host plant fitness in a complex
343 ecosystem.

344 Thus, these three interpretations which are not mutually exclusive could
345 complimentary contribute to explain the mosaic-like, spatial genetic structure of *Frankia*.
346 In future studies, phenotypes and functions of different OTUs detected in this study
347 should be investigated.

348

349 Spatial structure in local genetic communities of *Frankia*

350 The results also revealed a complex, mosaic-like, genetic structures of *Frankia* on a
351 single-host–population scale (spatial differentiation of *Frankia* OTU components; Fig 3)
352 that did not depend on geographic distance. The explanations mentioned in the above
353 section can also contribute to the understanding of the spatial mosaic-like patterns
354 observed in *Frankia* genetic communities. The heterogeneous spatial structure of the
355 interactions between genetically diverse hosts and rhizo-microorganisms can exert
356 selective pressure resulting in the spatial differentiation of *Frankia* communities. In fact,
357 we detected a genetic differentiation of the alder host not only among the studied riparian

358 areas but also within each area (Kagiya et al. 2018), as well as the spatial genetic structure
359 of *Frankia*. However, a part of patterns in genetic structure of *Frankia* were inconsistent
360 with the pattern of host genetic structure. For example, *Frankia* compositions were
361 different between BT and SE area (Fig. 5), while *A. hirsuta* populations were closely-
362 related. BT area is completely covered with natural forest stands but SE area is close to
363 agriculture field and its riverside landscape is artificially modified. The differences in
364 abiotic and biotic environments could affect the selection outcomes (e.g., $G \times G \times E$:
365 genotype-by-genotype-by-environment interactions), which may contribute the
366 observed mosaic-like structures of the *Frankia* communities.

367 Furthermore, the dispersion processes of *Frankia* may play a key role in generating
368 these spatial patterns. The significant genetic differentiation of *Frankia* among riparian
369 areas (Table 1) may be due to the dispersal of *Frankia* bacteria by the waterways (Arveby
370 and Huss-Danell 1988; Huss-Danell et al. 1997). In addition, massive snow-melt in the
371 study site (snow depth: > 200 cm) may also drive soil bacteria dispersion by transporting
372 soil components along the complex river landscape. Previous studies suggested that
373 herbivorous mammals (Chaia et al. 2012), birds (Paschke and Dawson 1993), and
374 invertebrates such as earthworms (Reddell and Spain 1991) can also drive the dispersion
375 of *Frankia*, carrying their propagules. *Frankia* propagules did not lose their activity to
376 infect their hosts despite going through the digestive tracts of such animals (Burleigh and
377 Dawson 1995; Chaia et al. 2012). Thus, the genetic mosaic-like structure can be, at least
378 partially, the result of both biotic (e.g., deer, birds, and earthworms) and abiotic dispersion
379 processes (snowmelt and waterways).

380 To our knowledge, ours is the first study that demonstrated spatial heterogeneity in
381 genetic diversity and composition of *Frankia* bacteria in a single-host-population scale.

382 Our findings suggest that actinorhizal host individuals can interact with different *Frankia*
383 strains within a population. The interactions with different genotypes of mutualistic
384 bacteria widely influences phenotypes of host plants (Ballhorn et al. 2017; Barrett et al.
385 2012; Dean et al. 2014; Miller and Sirois 1982; Pahua et al. 2018; Prat 1989; Robinson
386 et al. 2000). The variation in mutualistic partnerships on small spatial scales could
387 increase the heterogeneity of ecosystem processes and/or associated community
388 dynamics in forest ecosystems. Understanding genetic structure of nitrogen-fixing
389 bacterial symbionts holds the key to elucidating these dynamics in forest ecosystems.
390 Further research is required to shed more light on the mechanisms that create the spatial
391 heterogeneity in genetic diversity and composition of actinorhizal symbionts on a local
392 scale.

393

394 **Acknowledgement**

395 We thank Rei Sakai, Michio Ishihara, Takashi Hara, Yoshiaki Takahashi,
396 Masayuki Watarai, Wataru Mamiya, Yuri Yabuhara, and members of Uryu Experimental
397 Forest, Hokkaido University, for their field assistance. This research was supported by
398 Grant-in-Aid for JSPS Research Fellow to SK (16J03194), Young Scientists A
399 (16H0617908) and Scientific Research B (19H0297409) to SU.

400

401 **Author contribution**

402 SK and SU designed and conducted the investigation, performed the molecular
403 analysis, analyzed the data, and wrote the manuscript.

404

405 **Data availability statement**

406 The sequence data are deposited at DDBJ with accession numbers of LC482655-
407 LC482672.

408 **References**

409

410 Ager AA, Heilman PE, Stettler RF (1993) Genetic variation in red alder (*Alnus rubra*) in
411 relation to native climate and geography. *Can J Forest Res* 23:1930–1939.
412 <https://doi.org/10.1139/x93-243>

413 Anderson MD, Ruess RW, Myrold DD, Taylor DL (2009) Host species and habitat affect
414 nodulation by specific *Frankia* genotypes in two species of *Alnus* in interior Alaska.
415 *Oecologia* 160:619–630. <https://doi.org/10.1007/s00442-009-1330-0>

416 Anderson MD, Taylor DL, Ruess RW (2013) Phylogeny and assemblage composition of
417 *Frankia* in *Alnus tenuifolia* nodules across a primary successional sere in interior
418 Alaska. *Mol Ecol* 22:3864–3877. <https://doi.org/10.1111/mec.12339>

419 Archetti M, Úbeda F, Fudenberg D, Green J, Pierce NE, Yu DW (2011) Let the right one
420 in: a microeconomic approach to partner choice in mutualisms. *Am Nat* 177:75–85.
421 <https://doi.org/10.1086/657622>

422 Arveby AS, Huss-Danell K (1988) Presence and dispersal of infective *Frankia* in peat
423 and meadow soils in Sweden. *Biol Fert Soils* 6:39–44.
424 <https://doi.org/10.1007/BF00257918>

425 Baker DD (1987) Relationships among pure cultured strains of *Frankia* based on host
426 specificity. *Physiol Plantarum* 70:245–248. <https://doi.org/10.1111/j.1399-3054.1987.tb06139.x>

428 Ballhorn DJ, Elias JD, Balkan MA, Fordyce RF, Kennedy PG (2017) Colonization by
429 nitrogen-fixing *Frankia* bacteria causes short-term increases in herbivore
430 susceptibility in red alder (*Alnus rubra*) seedlings. *Oecologia* 184:497–506.
431 <https://doi.org/10.1007/s00442-017-3888-2>

432 Barrett LG, Broadhurst LM, Thrall PH (2012) Geographic adaptation in plant-soil
433 mutualisms: tests using *Acacia* spp. and rhizobial bacteria. *Funct Ecol* 26:457–468.
434 <https://doi.org/10.1111/j.1365-2435.2011.01940.x>

435 Ben Tekaya S, Guerra T, Rodriguez D, Dawson JO, Hahn D (2018) *Frankia* diversity in
436 host plant root nodules is independent of abundance or relative diversity of *Frankia*
437 populations in corresponding rhizosphere soils. *Appl Environ Microb* 84:1–11.
438 <https://doi.org/10.1128/AEM.02248-17>

439 Benson DR, Dawson JO. (2007) Recent advances in the biogeography and genecology of
440 symbiotic *Frankia* and its host plants. *Physiol Plantarum* 130:318–330.
441 <https://doi.org/10.1111/j.1399-3054.2007.00934.x>

442 Benson DR, Hanna D. (1983) *Frankia* diversity in an alder stand as estimated by sodium
443 dodecyl sulfate–polyacrylamide gel electrophoresis of whole-cell proteins. *Can J*
444 *Botany* 61:2919–2923. <https://doi.org/10.1139/b83-325>

445 Berry AM, Mendoza-Herrera A, Guo Y et al (2011) New perspectives on nodule nitrogen
446 assimilation in actinorhizal symbioses. *Funct Plant Biol* 38:645–652.
447 <https://doi.org/10.1071/FP11095>

448 Burleigh SH, Dawson JO (1995) Spores of *Frankia* strain HFPCc13 nodulate *Casuarina*
449 *equisetifolia* after passage through the digestive tracts of captive parakeets
450 (*Melopsittacus undulatus*). *Can J Botany* 73:1527–1530.
451 <https://doi.org/10.1139/b95-165>

452 Caldwell BE (1966) Inheritance of a strain-specific ineffective nodulation in soybeans.
453 *Crop Sci* 6:427–428. <https://doi.org/10.2135/cropsci1966.0011183X000600050010x>

- 454 Chaia EE, Sosa MC, Raffaele E (2012) Vertebrate faeces as sources of nodulating *Frankia*
455 in Patagonia. *Symbiosis* 56:139–145. <https://doi.org/10.1007/s13199-012-0169-z>
- 456 Chen Z, Li J. (2004) Phylogenetics and biogeography of *Alnus* (Betulaceae) inferred from
457 sequences of nuclear ribosomal DNA ITS region. *Int J Plant Sci*, 165:325–335.
458 <https://doi.org/10.1086/382795>
- 459 Clawson ML, Caru M, Benson DR (1998) Diversity of *Frankia* strains in root nodules of
460 plants from the families Elaeagnaceae and Rhamnaceae. *Appl Environ Microb*
461 64:3539–3543. <https://doi.org/10.5301/HIP.2012.9281>
- 462 Clawson ML, Gawronski J, Benson DR. (1999) Dominance of *Frankia* strains in stands
463 of *Alnus incana* subsp. *rugosa* and *Myrica pensylvanica*. *Can J Botany*, 77:1203–
464 1207. <https://doi.org/10.1139/b99-070>
- 465 Cunnings A, Johnson E, Martin Y (2016) Fluvial seed dispersal of riparian trees:
466 Transport and depositional processes. *Earth Surf Proc Land* 41:615–625.
467 <https://doi.org/10.1002/esp.3850>
- 468 Dean J, Mescher M, De Moraes C (2014) Plant dependence on rhizobia for nitrogen
469 influences induced plant defenses and herbivore performance. *Int J Mol Sci*
470 15:1466–1480. <https://doi.org/10.3390/ijms15011466>
- 471 Dillon JT, Baker D (1982) Variations in nitrogenase activity among pure-cultured *Frankia*
472 strains tested on actinorhizal plants as an indication of symbiotic compatibility. *New*
473 *Phytol* 92:215–219. <https://doi.org/10.1111/j.1469-8137.1982.tb03378.x>
- 474 Du D, Baker DD (1992) Actinorhizal host-specificity of Chinese *Frankia* strains. *Plant*
475 *Soil* 144:113–116. <https://doi.org/10.1007/BF00018851>
- 476 Ellison AM, Bank MS, Clinton BD et al (2005) Loss of foundation species: consequences
477 for the structure and dynamics of forested ecosystems. *Front Ecol Environ* 3:479–
478 486. [https://doi.org/10.1890/1540-9295\(2005\)003\[0479:LOFSCF\]2.0.CO;2](https://doi.org/10.1890/1540-9295(2005)003[0479:LOFSCF]2.0.CO;2)
- 479 Ellison AM, Barker-Plotkin AA, Foster DR, Orwig DA (2010) Experimentally testing the
480 role of foundation species in forests: the Harvard Forest Hemlock Removal
481 Experiment. *Methods Ecol Evol* 1:168–179. <https://doi.org/10.1111/j.2041-210x.2010.00025.x>
- 483 Ferriere R, Bronstein JL, Rinaldi S, Law R, Gauduchon M (2002) Cheating and the
484 evolutionary stability of mutualisms. *P Roy Soc Lond B Bio* 269:773–780.
485 <https://doi.org/10.1098/rspb.2001.1900>
- 486 Hahn D, Starrenburg MJC, Akkermans ADL (1988) Variable compatibility of cloned
487 *Alnus glutinosa* ecotypes against ineffective *Frankia* strains. *Plant Soil* 107:233–243.
488 <https://doi.org/10.1007/BF02370552>
- 489 Hayashi M, Saeki Y, Haga M, Harada K, Kouchi H, Umehara Y (2012) *Rj* (*rj*) genes
490 involved in nitrogen-fixing root nodule formation in soybean. *Breeding Sci* 61:544–
491 553. <https://doi.org/10.1270/jsbbs.61.544>
- 492 He XH, Chen LG, Hu XQ, Asghar S. (2004) Natural diversity of nodular microsymbionts
493 of *Myrica rubra*. *Plant Soil*, 262:229–239.
494 <https://doi.org/10.1023/B:PLSO.0000037045.42440.1d>
- 495 Heath KD, Stinchcombe JR (2014) Explaining mutualism variation: a new evolutionary
496 paradox? *Evolution* 68:309–317. <https://doi.org/10.1111/evo.12292>
- 497 Heath KD, Tiffin P (2007) Context dependence in the coevolution of plant and rhizobial
498 mutualists. *P Roy Soc B Biol Sci* 274:1905–1912.
499 <https://doi.org/10.1098/rspb.2007.0495>

- 500 Hoeksema JD, Kummel M (2003) Ecological persistence of the plant - mycorrhizal
501 mutualism: a hypothesis from species coexistence theory. *Am Nat* 162:S40-S50.
502 <https://doi.org/10.1086/378644>
- 503 Hooker JE, Wheeler CT (1987) The effectivity of *Frankia* for nodulation and nitrogen
504 fixation in *Alnus rubra* and *A. glutinosa*. *Physiol Plantarum* 70:333-341.
505 <https://doi.org/10.1111/j.1399-3054.1987.tb06152.x>
- 506 Huguet V, Batzli JM, Zimpfer JF, Normand P, Dawson JO, Fernandez MP. (2001)
507 Diversity and specificity of *Frankia* strains in nodules of sympatric *Myrica gale*,
508 *Alnus incana*, and *Shepherdia canadensis* determined by *rrs* gene polymorphism.
509 *Appl Environ Microb*, 67:2116-2122. [https://doi.org/10.1128/AEM.67.5.2116-](https://doi.org/10.1128/AEM.67.5.2116-2122.2001)
510 [2122.2001](https://doi.org/10.1128/AEM.67.5.2116-2122.2001)
- 511 Huss-Danell K, Uliassi D, Renberg I (1997) River and lake sediments as sources of
512 infective *Frankia* (*Alnus*). *Plant Soil* 197:35-39.
513 <https://doi.org/10.1023/A:1004268931699>
- 514 Jiabin H, Zheyang Z, Guanxiong C, Huichang L (1985) Host range of *Frankia* endophytes.
515 *Plant Soil* 87:61-65. <https://doi.org/10.1007/BF02277648>
- 516 Kagiya S, Yasugi M, Kudoh H, Nagano AJ, Utsumi S (2018) Does genomic variation in
517 a foundation species predict arthropod community structure in a riparian forest? *Mol*
518 *Ecol* 27:1284-1295. <https://doi.org/10.1111/mec.14515>
- 519 Kennedy PG, Weber, MG, Bluhm AA (2010) *Frankia* bacteria in *Alnus rubra* forests:
520 genetic diversity and determinants of assemblage structure. *Plant Soil* 335:479-492.
521 <https://doi.org/10.1007/s11104-010-0436-9>
- 522 Khan A, Myrold DD, Misra AK. (2007) Distribution of *Frankia* genotypes occupying
523 *Alnus nepalensis* nodules with respect to altitude and soil characteristics in the
524 Sikkim Himalayas. *Physiol Plantarum*, 130:364-371. [https://doi.org/10.1111/j.1399-](https://doi.org/10.1111/j.1399-3054.2006.00872.x)
525 [3054.2006.00872.x](https://doi.org/10.1111/j.1399-3054.2006.00872.x)
- 526 Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions
527 through comparative studies of nucleotide sequences. *J Mol Evol* 16:111-120.
528 <https://doi.org/10.1007/BF01731581>
- 529 King RA, Ferris C (1998) Chloroplast DNA phylogeography of *Alnus glutinosa* (L.)
530 Gaertn. *Mol Ecol* 7:1151-1161. <https://doi.org/10.1046/j.1365-294x.1998.00432.x>
- 531 Kucho K, Hay A, Normand P (2010) The determinants of the actinorhizal symbiosis.
532 *Microbes Environ* 25:241-252. <https://doi.org/10.1264/jsme2.ME10143>
- 533 Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics
534 Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol* 33:1870-1874.
535 <https://doi.org/10.1093/molbev/msw054>
- 536 Lawrence DB, Schoenike RE, Quispel A, Bond G (1967) The role of *Dryas drummondii*
537 in vegetation development following ice recession at Glacier Bay, Alaska, with
538 special reference to its nitrogen fixation by root nodules. *J Ecol* 55:793-813.
539 <https://doi.org/10.2307/2258426>
- 540 Li W, Godzik A (2006) Cd-hit: a fast program for clustering and comparing large sets of
541 protein or nucleotide sequences. *Bioinformatics* 22:1658-1659.
542 <https://doi.org/10.1093/bioinformatics/btl158>
- 543 Markham JH (2008) Variability of nitrogen-fixing *Frankia* on *Alnus* species. *Botany* 86:
544 501-510. <https://doi.org/10.1139/B08-023>

545 Miller RW, Sirois JC (1982) Relative efficacy of different alfalfa cultivar-*Rhizobium*
546 *meliloti* strain combinations for symbiotic nitrogen fixation. *Appl Environ Microb*
547 43:764–768. <http://www.ncbi.nlm.nih.gov/pubmed/16345986>

548 Mirza BS, Welsh A, Rasul G, Rieder JP, Paschke MW, Hahn D (2009) Variation in
549 *Frankia* populations of the *Elaeagnus* host infection group in nodules of six host
550 plant species after inoculation with soil. *Microb Ecol* 58:384–393.
551 <https://doi.org/10.1007/s00248-009-9513-0>

552 Mishra AK, Singh PK, Singh P et al (2015) Phylogeny and evolutionary genetics of
553 *Frankia* strains based on 16S rRNA and *nifD*-K gene sequences. *J Basic Microb*
554 55:1013–1020. <https://doi.org/10.1002/jobm.201400914>

555 Normand P, Lapierre P, Tisa LS et al (2007) Genome characteristics of facultatively
556 symbiotic *Frankia* sp. strains reflect host range and host plant biogeography.
557 *Genome Res* 17:7–15. <https://doi.org/10.1101/gr.5798407>

558 Normand P, Orso S, Cournoyer B et al (1996) Molecular phylogeny of the genus *Frankia*
559 and related genera and emendation of the family Frankiaceae. *Int J Syst Bacteriol*
560 46:1–9. <https://doi.org/10.1099/00207713-46-1-1>

561 Nouioui I, Ghodhbane-Gtari F, Fernandez MP, Boudabous A, Normand P, Gtari M (2014)
562 Absence of cospeciation between the uncultured *Frankia* microsymbionts and the
563 disjunct actinorhizal *Coriaria* species. *BioMed Res Int* 2014:1-9.
564 <https://doi.org/10.1155/2014/924235>

565 Nyeko P, Edwards-Jones G, Day RK (2002) Population dynamics of herbivorous insects
566 and potential arthropod natural enemies on *Alnus* species in Kabale district, Uganda.
567 *Agroforest Syst* 56:213–224. <https://doi.org/10.1023/A:1021376414975>

568 Oksanen J, Blanchet FG, Friendly M et al (2019). *vegan*: Community ecology package.
569 R Package Version 2.5-6. Retrieved from <https://github.com/vegandevs/vegan>

570 Pahua VJ, Stokes PJN, Hollowell AC et al (2018). Fitness variation among host species
571 and the paradox of ineffective rhizobia. *J Evolution Biol* 31:599–610.
572 <https://doi.org/10.1111/jeb.13249>

573 Paschke MW, Dawson JO (1993) Avian dispersal of *Frankia*. *Can J Botany* 71:1128–
574 1131. <https://doi.org/10.1139/b93-132>

575 Peakall R, Smouse PE. (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic
576 software for teaching and research--an update. *Bioinformatics* 28:2537–2539.
577 <https://doi.org/10.1093/bioinformatics/bts460>

578 Pebesma E, Bivand R, Rowlingson B et al (2018). *sp*: Classes and methods for spatial
579 data. R Package Version 1.3-1. Retrieved from <https://github.com/edzer/sp>

580 Pokharel A, Mirza BS, Dawson JO, Hahn D. (2011). *Frankia* populations in soil and root
581 nodules of sympatrically grown *Alnus* taxa. *Microb Ecol* 61:92–100.
582 <https://doi.org/10.1007/s00248-010-9726-2>

583 Pölme S, Bahram M, Kõljalg U, Tedersoo L (2014) Global biogeography of *Alnus*-
584 associated *Frankia* actinobacteria. *New Phytol* 204:979–988.
585 <https://doi.org/10.1111/nph.12962>

586 Pozzi AC, Roy M, Nagati M et al (2018) Patterns of diversity, endemism and
587 specialization in the root symbiont communities of alder species on the island of
588 Corsica. *New Phytol* 219:336–349. <https://doi.org/10.1111/nph.14996>

589 Pozzi AC, Bautista-Guerrero HH, Abby SS et al (2018a). Robust *Frankia* phylogeny,
590 species delineation and intraspecific diversity based on multi-locus sequence analysis
591 (MLSA) and single-locus strain typing (SLST) adapted to a large sample size. *Syst*

592 Appl Microbiol 41:311–323. <https://doi.org/10.1016/j.syapm.2018.03.002>
 593 Pozzi AC, Bautista-Guerrero HH, Nouioui I et al (2015). In-plant sporulation phenotype:
 594 a major life history trait to understand the evolution of *Alnus*-infective *Frankia*
 595 strains. Environ Microbiol 17:3125–3138. <https://doi.org/10.1111/1462-2920.12644>
 596 Pozzi AC, Roy M, Nagati M et al (2018b). Patterns of diversity, endemism and
 597 specialization in the root symbiont communities of alder species on the island of
 598 Corsica. New Phytol 219:336–349. <https://doi.org/10.1111/nph.14996>
 599 Prat D (1989) Effects of some pure and mixed *Frankia* strains on seedling growth in
 600 different *Alnus* species. Plant Soil 113:31–38. <https://doi.org/10.1007/BF02181918>
 601 Reddell P, Spain AV (1991) Transmission of infective *Frankia* (Actinomycetales)
 602 propagules in casts of the endogeic earthworm *Pontoscolex corethrurus*
 603 (Oligochaeta: Glossoscolecidae). Soil Biol Biochem 23:775–778.
 604 [https://doi.org/10.1016/0038-0717\(91\)90148-D](https://doi.org/10.1016/0038-0717(91)90148-D)
 605 Ren BQ, Xiang XG, Chen ZD. (2010) Species identification of *Alnus* (Betulaceae) using
 606 nrDNA and cpDNA genetic markers. Mol Ecol Resour 10:594–605.
 607 <https://doi.org/10.1111/j.1755-0998.2009.02815.x>
 608 Ridgway KP, Marland LA, Harrison AF, Wright J, Young JPW, Fitter AH (2004)
 609 Molecular diversity of *Frankia* in root nodules of *Alnus incana* grown with inoculum
 610 from polluted urban soils. FEMS Microbiol Ecol 50:255–263.
 611 <https://doi.org/10.1016/j.femsec.2004.07.002>
 612 Robinson KO, Beyene DA, van Berkum P, Knight-Mason R, Bhardwaj HL (2000)
 613 Variability in plant-microbe interaction between *Lupinus* lines and *Bradyrhizobium*
 614 strains. Plant Sci 159:257–264. [https://doi.org/10.1016/S0168-9452\(00\)00345-9](https://doi.org/10.1016/S0168-9452(00)00345-9)
 615 Roy M, Pozzi AC, Gareil R et al (2017) Alder and the golden fleece: high diversity of
 616 *Frankia* and ectomycorrhizal fungi revealed from *Alnus glutinosa* subsp. *barbata*
 617 roots close to a tertiary and glacial refugium. PeerJ 5:e3479.
 618 <https://doi.org/10.7717/peerj.3479>
 619 Sellstedt A, Huss-Danell K, Ahlqvist A (1986) Nitrogen fixation and biomass production
 620 in symbioses between *Alnus incana* and *Frankia* strains with different hydrogen
 621 metabolism. Physiol Plantarum 66:99-107. <https://doi.org/10.1111/j.1399-3054.1986.tb01240.x>
 622
 623 Simonet P, Bosco M, Chapelon C, Moiroud A, Normand P. (1994) Molecular
 624 characterization of *Frankia* microsymbionts from spore-positive and spore-negative
 625 nodules in a natural alder stand. Appl Environ Microbiol 60:1335–1341.
 626 <https://aem.asm.org/content/60/4/1335/article-info>
 627 Simonet P, Thi Le N, Moiroud A, Bardin R. (1989) Diversity of *Frankia* strains isolated
 628 from a single alder stand. Plant Soil 118:13–22.
 629 <https://doi.org/10.1007/BF02232786>
 630 Smith KP, Goodman RM (1999) Host variation for interactions with beneficial plant-
 631 associated microbes. Annu Rev Phytopathol 37:473–491.
 632 <https://doi.org/10.1146/annurev.phyto.37.1.473>
 633 Thamer S, Schädler M, Bonte D, Ballhorn DJ (2011) Dual benefit from a belowground
 634 symbiosis: nitrogen fixing rhizobia promote growth and defense against a specialist
 635 herbivore in a cyanogenic plant. Plant Soil 341:209–219.
 636 <https://doi.org/10.1007/s11104-010-0635-4>
 637 Torrey JG (1987) Endophyte sporulation in root nodules of actinorhizal plants. Physiol
 638 Plantarum, 70:279–288. <https://doi.org/10.1111/j.1399-3054.1987.tb06145.x>

- 639 Vanden Heuvel BD, Benson DR, Bortiri E, Potter D. (2004) Low genetic diversity among
640 *Frankia* spp. strains nodulating sympatric populations of actinorhizal species of
641 Rosaceae, *Ceanothus* (Rhamnaceae) and *Datisca glomerata* (Datiscaceae) west of
642 the Sierra Nevada (California). *Can J Microbiol* 50:989–1000.
643 <https://doi.org/10.1139/w04-079>
- 644 Wheeler CT, Akkermans ADL, Berry AM (2008) *Frankia* and actinorhizal plants: a
645 historical perspective. In: Pawlowski K, Newton WE, eds. Nitrogen-fixing
646 actinorhizal symbioses. Dordrecht, The Netherlands: Springer US, 1–24.
- 647 Wickneswari R, Norwati M (1993) Genetic diversity of natural-populations of *Acacia*
648 *auriculiformis*. *Aust J Bot* 41: 65-77. <https://doi.org/10.1071/BT9930065>
- 649 Wilcox DA, Cowan DA (2016) Diversity of *Frankia* in root nodules of six *Morella* sp.
650 from the Cape flora of South Africa. *Plant Soil* 406:375–388.
651 <https://doi.org/10.1007/s11104-016-2881-6>
- 652 Yamakawa T, Hussain AKMA, Ishizuka J (2003) Soybean preference for *Bradyrhizobium*
653 *japonicum* for nodulation. *Soil Sci Plant Nutr* 49:835–841.
654 <https://doi.org/10.1080/00380768.2003.10410345>
655

656

Tables

657

Table 1. Explanation of genetic structure by study areas/sites based on AMOVA.

Source	df	Estimate	P
Among areas	4	1.463	0.015
Among sites	12	2.521	0.012
Within sites	184	47.552	< 0.001

658

659 **Figure legends**

660 **Fig 1.** Map of the sampling points in the Uryu Experimental Forest of Hokkaido
661 University, northern Hokkaido, Japan, where *A. hirsuta* seedlings root nodules were
662 collected. Different colors indicate different areas: (a) overall map, (b–f) individual areas
663 (b: UT; c: DRW; d: BT; e: SE; f: DRE). Marker shapes in magnified map areas (b-f)
664 indicate sites within each area.

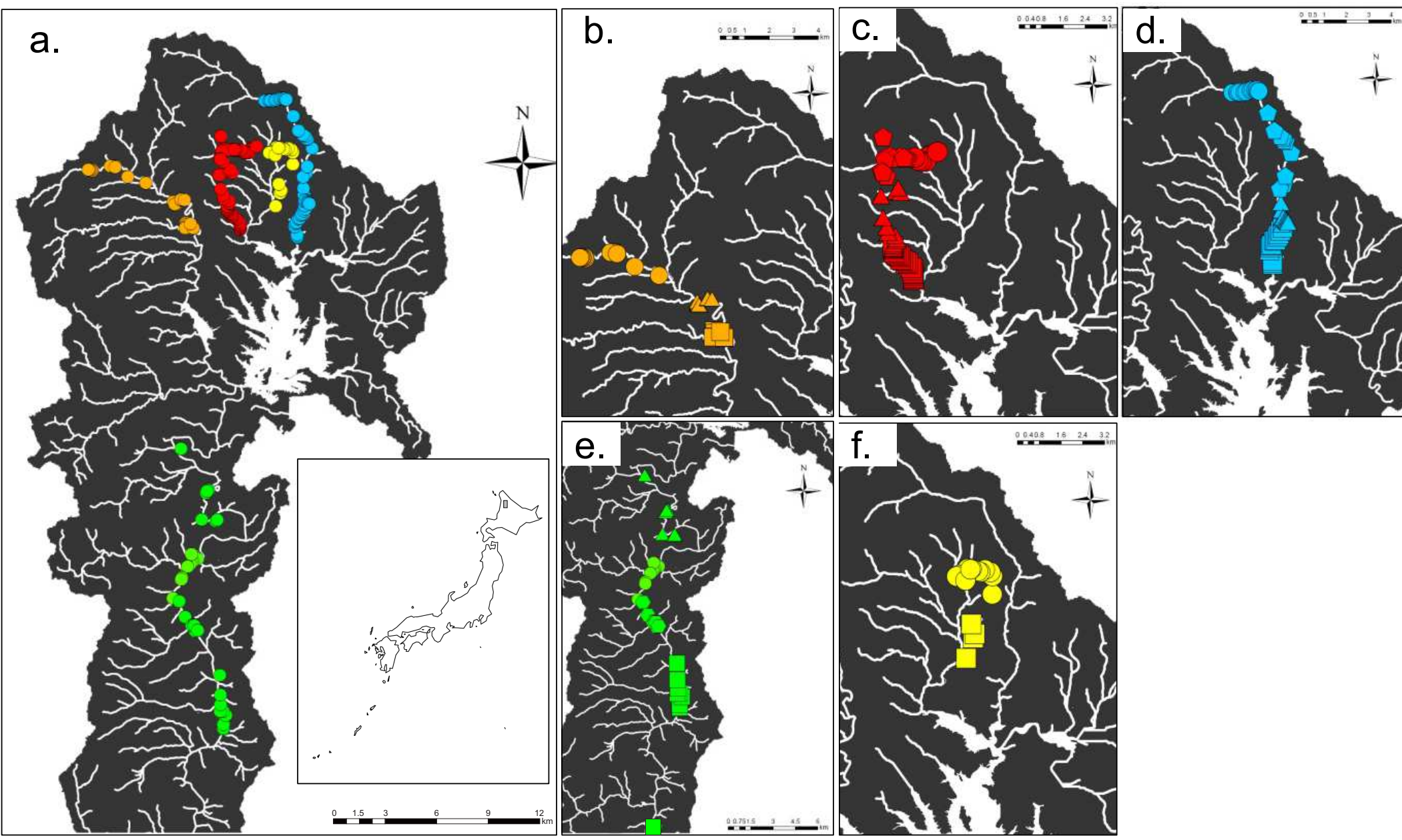
665
666 **Fig 2.** Phylogenetic tree based on the *nifD*-K spacer region in *Frankia*. A maximum-
667 likelihood phylogeny was generated based on 97 sequences of the *nifD*-K locus, obtained
668 from three subspecies of *Alnus incana* (ssp. *hirsuta*: operational taxonomic units ‘OTUs_’
669 in this study; ssp. *rubra*: ‘AR_’; ssp. *tenuifolia*: ‘AT_’), *A. viridis* (‘AV_’), nine varieties
670 of *Myrica rubra* (var. *biji*: ‘Mbj_’; var. *baimei*: ‘Mbm_’; var. *dongkui*: ‘Mdk_’; var. *muye*:
671 ‘Mmy_’; var. *shuimei*: ‘Msm_’; var. *wandao*: ‘Mwd_’; var. *wumei*: ‘Mwy_’; var. *yangliu*:
672 ‘Myl_’; var. *zaoda*: ‘Mdz_’), and the ACN14a strain as an *Alnus* infection clade. Other
673 sequences obtained from *Hippöphae salicifolia* (‘Hsli_’), three *Coriaria* species (*C.*
674 *myrtifolia*: ‘Cm_’; *C. japonica*: ‘Cj_’; *C. arborea*: ‘Ca_’), *Elaeagnus* (‘EAN1pec’),
675 *Datisca glomerate*, and *Casuarina equisetifolia* (‘CcI3’) were also included in the
676 phylogeny as outgroups. These sequences were obtained from GenBank. The characters
677 in parentheses indicate accession numbers on GenBank. Branch labels indicate significant
678 bootstrap values. The tree is drawn to scale, with branch lengths measured according to
679 the number of substitutions per site.

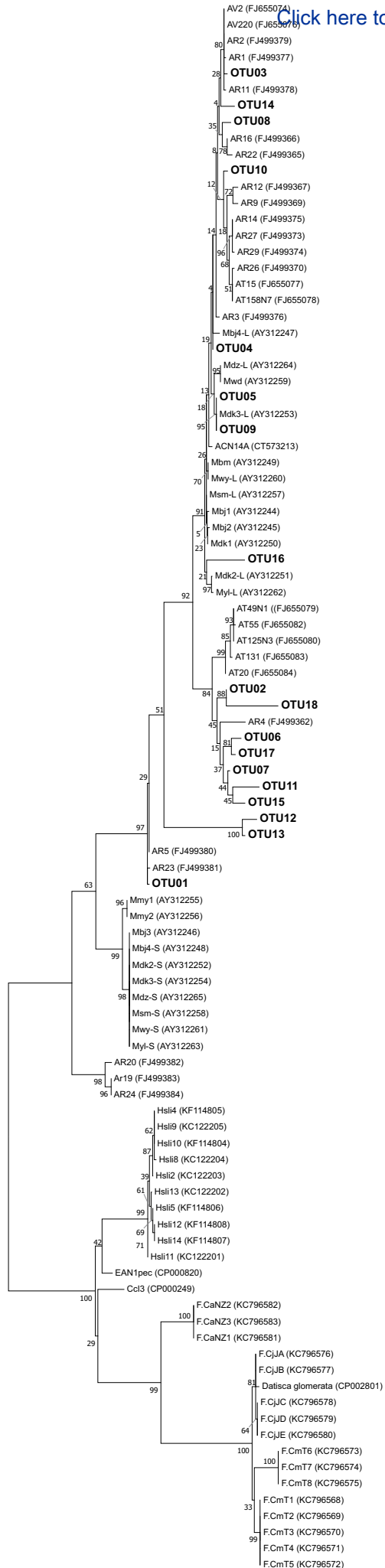
680
681 **Fig 3.** *Frankia* diversity along sampling size in each site. Different colors indicate
682 different areas

683
684 **Fig 4.** Relationships between *Frankia* operational taxonomic units (OTUs) and areas. The
685 phylogeny was generated using the maximum-likelihood method. Branch values indicate
686 significant bootstrap values. Circles indicate the presence of each OTU in each site. Circle
687 sizes represent numbers of OTUs in each site (see also Table 1).

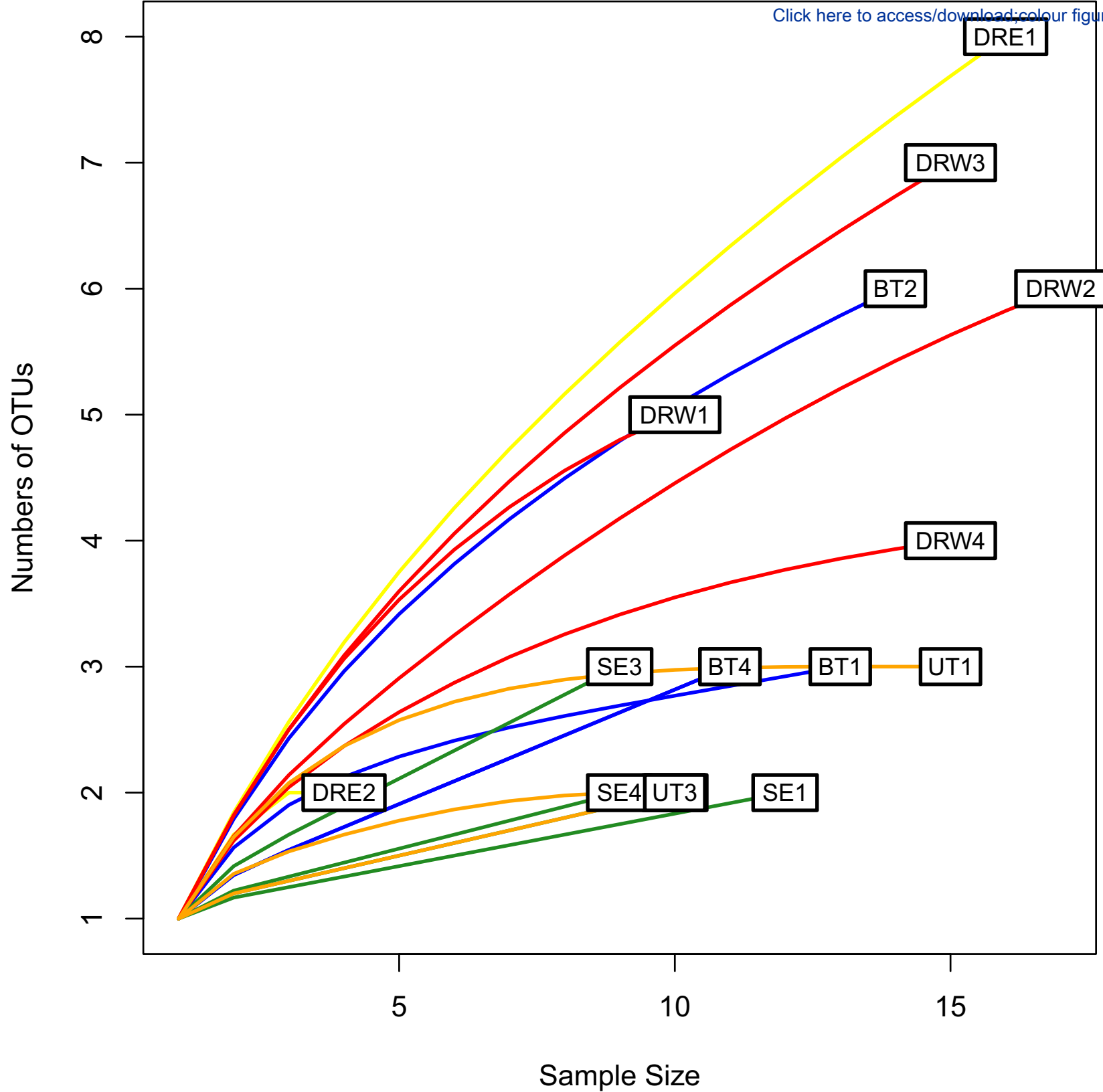
688
689 **Fig 5.** Non-metric multidimensional scaling (NMDS) of *Frankia* OTU compositions at
690 the site-levels. Colors of points indicate areas. The numbers in points indicate ID of sites.

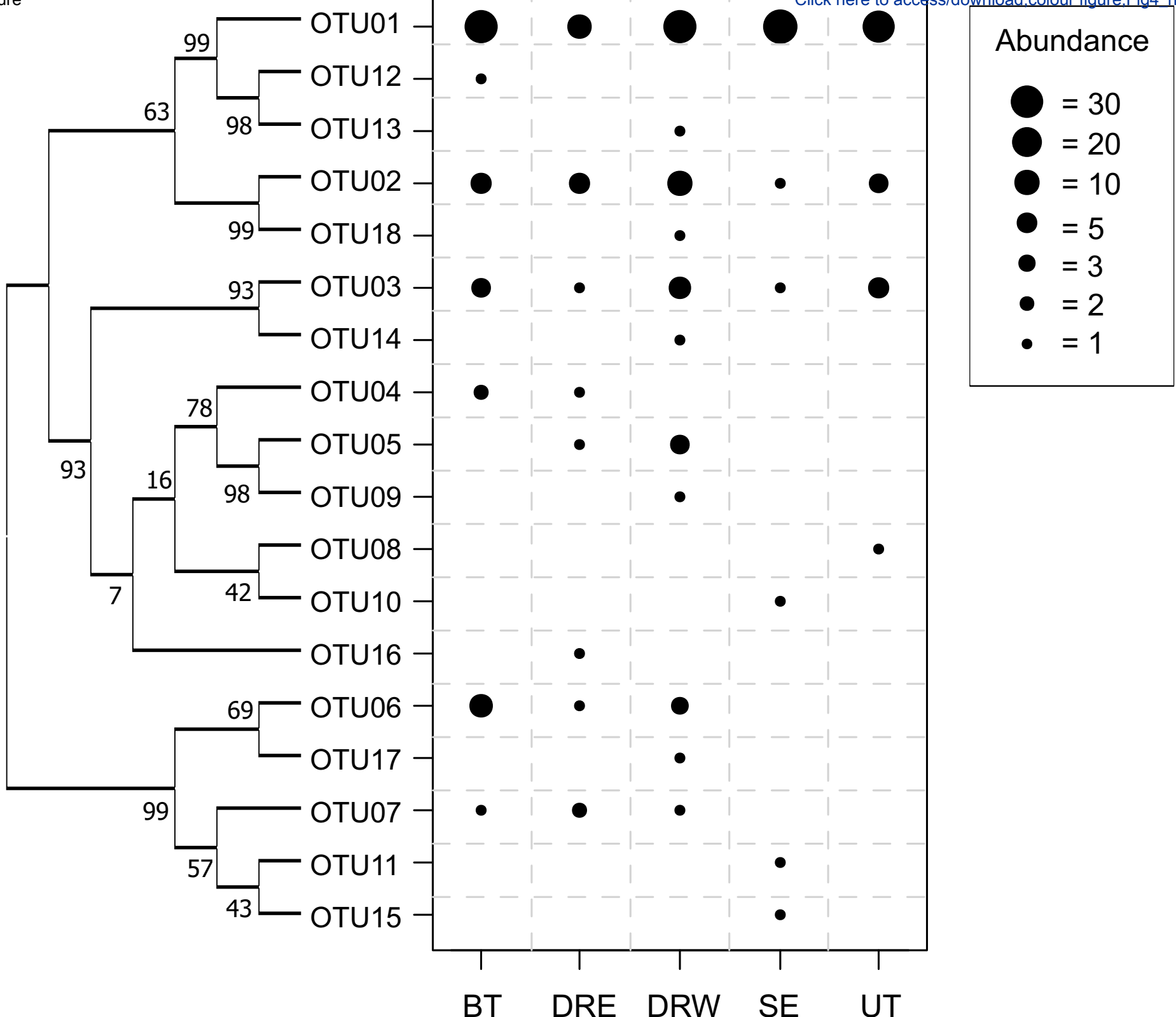
691

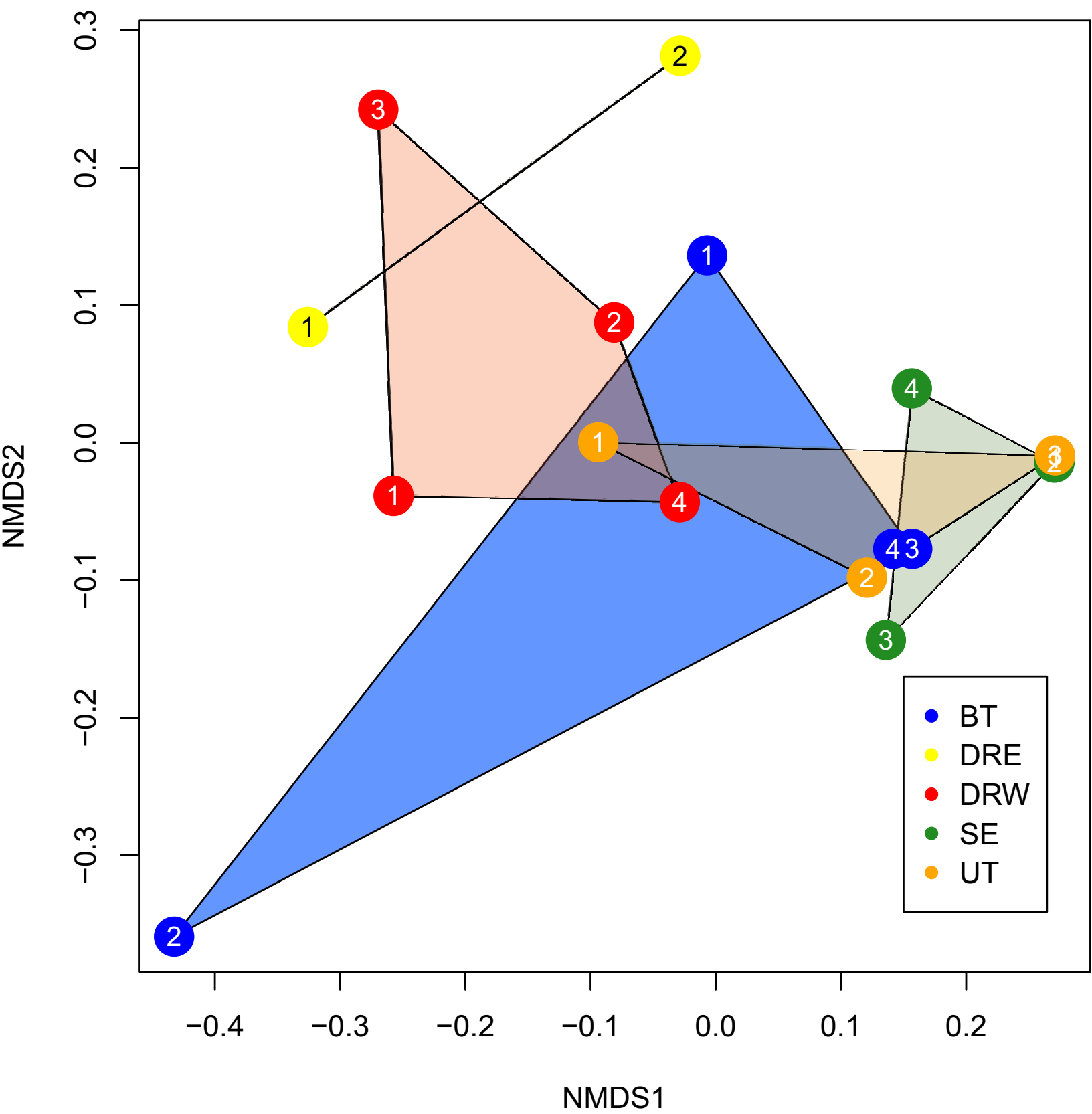




Alnus-infective clade







Article title: High genetic diversity of bacterial symbionts in a single host species population: an alder–Frankia system in the field

Journal name: Plant and Soil

Author names: Shinnosuke Kagiya¹, Shunsuke Utsumi²

Affiliation:

¹Graduate School of Environmental Science, Hokkaido University, North 10, West 5, Sapporo, Hokkaido 060-0810, Japan ²Field Science Center of Northern Biosphere, Hokkaido University, North 9, West 9, Sapporo, Hokkaido 060-0809, Japan

E-mail: Shinnosuke Kagiya (e-mail: slotapir-1035@fsc.hokudai.ac.jp)

Table S1. Numbers of OTUs obtained from each site.

Area	BT				DRE		DRW				SE				UT			Accession No.
Site No.	1	2	3	4	1	2	1	2	3	4	1	2	3	4	1	2	3	
OTU01	8	3	9	9	6	2	4	11	5	9	11	9	7	6	8	8	9	Ahi01 (LC482655)
OTU02	4	1	0	0	3	2	2	2	2	2	0	0	0	1	4	0	0	Ahi02 (LC482656)
OTU03	0	2	1	1	1	0	2	1	0	2	0	0	1	0	3	2	0	Ahi03 (LC482657)
OTU04	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	Ahi04 (LC482658)
OTU05	0	0	0	0	1	0	0	2	2	0	0	0	0	0	0	0	0	Ahi05 (LC482659)
OTU06	0	6	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	Ahi06 (LC482660)
OTU07	0	0	0	1	2	0	1	0	0	0	0	0	0	0	0	0	0	Ahi07 (LC482661)
OTU08	0	0	0	0	1	0	0	0	1	2	0	0	0	1	0	0	0	Ahi08 (LC482662)
OTU09	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Ahi09 (LC482663)
OTU10	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ahi10 (LC482664)
OTU11	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Ahi11 (LC482665)
OTU12	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Ahi12 (LC482666)
OTU13	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Ahi13 (LC482667)
OTU14	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Ahi14 (LC482668)
OTU15	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ahi15 (LC482669)
OTU16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Ahi16 (LC482670)
OTU17	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Ahi17 (LC482671)
OTU18	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Ahi18 (LC482672)
Total	13	14	11	11	16	4	10	17	15	15	12	10	9	9	15	10	10	

Table S2. Shannon diversity indices, evenness, richness and sample size of *Frankia* OTU compositions in each site and area and whole of the Uryu Experimental Forest.

	Region	Shannon	Evenness	Richness	Sample size
Site-level	BT1	0.8587	0.7817	3	13
	BT2	1.5367	0.8577	6	14
	BT3	0.6002	0.5463	3	11
	BT4	0.6002	0.5463	3	11
	DRE1	1.8080	0.8695	8	16
	DRE2	0.6931	1.0000	2	4
	DRW1	1.4708	0.9139	5	10
	DRW2	1.3157	0.7343	6	17
	DRW3	1.6792	0.8629	7	15
	DRW4	1.0776	0.7773	4	15
	SE1	0.2868	0.4138	2	12
	SE2	0.3251	0.4690	2	10
	SE3	0.6837	0.6224	3	9
	SE4	0.3488	0.5033	2	9
	UT1	1.0096	0.9190	3	15
	UT2	0.5004	0.7219	2	10
	UT3	0.3251	0.4690	2	10
	Area-level	BT	1.3153	0.6759	7
DRE		1.6923	0.8138	8	20
DRW		1.6392	0.6836	11	57
SE		0.5779	0.3226	6	40
UT		0.8678	0.6260	4	35
Whole forest		1.4373	0.4973	18	201

Fig S1. Phylogenetic tree based on the *nifD*-K spacer region in *Frankia*. The neighbor-joining phylogeny was generated based on 97 sequences of the *nifD*-K locus, obtained from three subspecies of *Alnus incana* (ssp. *hirsuta*: operational taxonomic units ‘OTUs_’ in this study; ssp. *rubra*: ‘AR_’; ssp. *tenuifolia*: ‘AT_’), *A. viridis* (‘AV_’), nine varieties of *Myrica rubra* (var. *biji*: ‘Mbj_’; var. *baimei*: ‘Mbm_’; var. *dongkui*: ‘Mdk_’; var. *muye*: ‘Mmy_’; var. *shuimei*: ‘Msm_’; var. *wandao*: ‘Mwd_’; var. *wumei*: ‘Mwy_’; var. *yangliu*: ‘Myl_’; var. *zaoda*: ‘Mdz_’), and the ACN14a strain as an *Alnus* infection clade. Other sequences obtained from *Hippöphae salicifolia* (‘Hsli_’), three *Coriaria* species (*C. myrtifolia*: ‘Cm_’; *C. japonica*: ‘Cj_’; *C. arborea*: ‘Ca_’), *Elaeagnus angustifolia* (EAN1pec), *Datisca glomerate* and *Casuarina equisetifolia* (‘Ccl3’) were also analyzed in the phylogeny as outgroups. These sequences were obtained from GenBank. The characters in parentheses indicated accession numbers on GenBank. Branch labels indicate significant bootstrap values. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

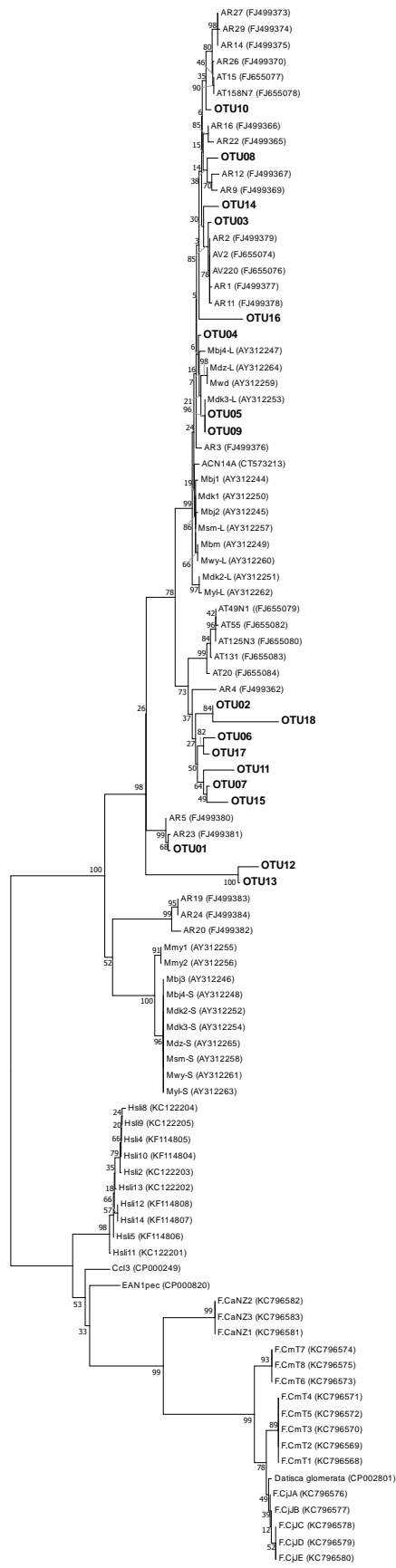


Fig S2. Phylogenetic tree based on all the *nifD*-K sequences of *Frankia*. A maximum-likelihood phylogeny was generated based on 280 sequences of the *nifD*-K locus, obtained from three subspecies of *Alnus incana* (ssp. *hirsuta*: operational taxonomic units ‘KS_’ in this study; ssp. *rubra*: ‘AR_’; ssp. *tenuifolia*: ‘AT_’), *A. viridis* (‘AV_’), nine varieties of *Myrica rubra* (var. *biji*: ‘Mbj_’; var. *baimei*: ‘Mbm_’; var. *dongkui*: ‘Mdk_’; var. *muye*: ‘Mmy_’; var. *shuimei*: ‘Msm_’; var. *wandao*: ‘Mwd_’; var. *wumei*: ‘Mwy_’; var. *yangliu*: ‘Myl_’; var. *zaoda*: ‘Mdz_’), and the ACN14a strain as an *Alnus* infection clade. Other sequences obtained from *Hippöphae salicifolia* (‘Hsli_’), three *Coriaria* species (*C. myrtifolia*: ‘Cm_’; *C. japonica*: ‘Cj_’; *C. arborea*: ‘Ca_’), *Elaeagnus* (‘EAN1pec’), *Datisca glomerata*, and *Casuarina equisetifolia* (‘CcI3’) were also included in the phylogeny as outgroups. These sequences were obtained from GenBank. The characters in parentheses indicate accession numbers on GenBank. Branch labels indicate significant bootstrap values. The tree is drawn to scale, with branch lengths measured according to the number of substitutions per site. We described OTU groups of each sequence.

