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

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Abstract

Aims

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

Methods

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

Results

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

Conclusions

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

Key words

Alnus hirsuta, *Frankia*, genetic diversity, local scale, *nif*D-K IGS region, nitrogen-fixing bacteria, root nodule symbiosis

Introduction

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

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

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

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

The most important reason why the knowledge of genetic diversity of *Frankia* is still limited on a small spatial scale may be the assumption that the genetic diversity of mutualistic partners is low on a local scale and in a single host species. The traditional mutualistic theory has suggested that the genetic diversity of mutualistic partners is constrained by hosts and could be decreased by the hosts' stabilizing mechanisms, such as partner choice and sanction (Archetti et al. 2011; Heath and Stinchcombe 2014).

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

However, the knowledge of genetic diversity of *Frankia* on a small spatial scale (e.g., seed dispersal range: many seeds of *Alnus* individuals dispersed within *c.* 140 m along a river (Cunnings et al. 2016)) should be required to understand outcomes of considerable variation in current actinorhizal ecological interactions, such as the effectiveness of the bacteria in growth and survival of the host plants. This is because effects of rhizobial mutualism often depend not only on genetic variation of mutualistic bacteria but also on intraspecific variation of host species (Caldwell 1966; Hayashi et al. 2012; Heath and Tiffin 2007; Yamakawa et al. 2003). For example, nodulation rates of *Frankia* strains could also differ among intraspecific host individuals (Hahn et al. 1988). In fact, large genetic variation in a host plant population, including actinorhizal and legume species, is also ubiquitous in a natural forest stand (Ager et al. 1993; Kagiya et al. 2018; King and Ferris 1998; Wickneswari and Norwati 1993) Our previous study revealed large genetic variation in a single *Alnus* species in a continuous natural forest (20 km × 70 km; Kagiya et al., 2018). Leaf traits, such as C:N ratio, leaf mass per area (LMA), and herbivory rate, varied with the genetic variation and localities with the forest. Therefore, we should pay attention to genetic diversity of rhizobial bacteria and its spatial heterogeneity on a small spatial scale, which may be crucial to determining outcomes of ecological interaction between rhizobial bacteria and actinorhizal host plants under natural ecosystem conditions.

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

Materials and Methods

Host species and nitrogen-fixing bacteria

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

Root nodule sampling

Our study sites are located in and around the Uryu Experimental Forest (44° 030–290N, 142° 010–200E) of Hokkaido University in northern Hokkaido, Japan. This experimental forest is a continuously mixed conifer–broadleaf forest of *c.* 25000 ha. One nodule was collected from each of the roots of 213 *A. hirsuta* seedlings (DBH: < 2 cm) from five riparian areas of the forest (BT, DRE, DRW, SE, and UT; the maximum distance between our areas was 43.5 km; Fig 1) because the host trees are mainly found along rivers, and streams are considered one of the primary dispersal pathways of *Frankia* bacteria (Arveby and Huss-Danell 1988; Huss-Danell et al. 1997). Seedlings from which we collected root nodules were selected from 17 sites from the riparian areas. Sampling root nodules from *A. hirsuta* seedlings would allow us to collect samples continuously from a whole forest and to estimate genetic diversity and composition of *Frankia* which interact with a single host plant population at present. Sampling was conducted from June to September 2016. The distance between sampling points in each site was more than 100 m. *Alnus hirsuta* is the only actinorhizal species in this forest.

Molecular Analyses

The collected nodules were surface-sterilized using 10% (v/v) Clorox bleach. DNA was extracted from root nodules using DNeasy Blood & Tissue Kit (Qiagen). The nodules were sliced using sterilized razor blades and crushed using sterilized homogenization sticks. The crushed lobes were heated to 37 °C for 30 min. with a 25 μl Proteinase K. Polymerase chain reaction (PCR) was performed to amplify a 496-bp fragment of a *nif*D-K intergenic spacer region with the *Frankia*-specific primer pair, *nif*D1310frGC (5’-CGC CAG ATG CAC TCC TGG GAC TAC T-3’), and *nif*KR331frGC (5’-CGG GCG AAG TGG CTG CGG AA-3’). We focused on intrageneric variation of *Frankia* based on the *nif*D-K IGS region. The genetic marker is considered to be one of the useful genetic markers for resolution at the species level of *Frankia* because the genetic region includes higher variable than ribosomal RNA (Anderson et al. 2009; Mishra et al. 2015). PCR amplification was performed as follows: 1 cycle at 95 °C for 2 min, followed by 35 cycles 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 successful PCR products were cleaned using an ExoSAP master mix containing 0.5 μl Exonuclease I (TaKaRa), 0.5 μl Shrimp Alkaline Phosphatase (SAP; New England BioLabs), and 2.0 μl sterile deionized water. Incubation using a thermal cycler was conducted with ExoSAP at 37 °C for 20 min and at 80 °C for 15 min. These products were sequenced with an automated sequencer (3730xl DNA Analyzer, Applied Biosystems). DNA sequence chromatograms were manually checked using FinchTV

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

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

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

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

Analysis of *Frankia* diversity

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

To consider spatial autocorrelation in the *Frankia* OTU compositions, we calculated spatial distance among sites and areas. Spatial distance was calculated based on location of each site/area with Great Circle distance method, using the R package sp 1.3-1(Pebesma et al. 2018). The location was calculated centroid location as the averaged latitude and longitude of each *A. hirsuta* seedling. Spatial autocorrelation of *Frankia* compositions was analyzed by Mantel tests with 9,999 permutations. These tests were performed using the vegan package.

Results

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

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

To illustrate differences in *Frankia* OTU diversity (i.e., the total number of OTUs in each site) among the sites with the standardization of sample size, we generated a rarefaction curve for each site (Fig 3). OTU diversity was greater in BT, DRE, and DRW areas than in SE and UT areas. While the rarefaction curves of SE and UT areas show the saturation of the total OTU number, those of BT, DRE and DRW areas indicated there was likely to be still undetected OTUs in each area.

Differences in *Frankia* composition

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

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

Spatial genetic structure of *Frankia*

The AMOVA indicated a significant genetic differentiation in *Frankia* genetic communities among riparian areas and sites (Table 1). In addition, no significant correlations were detected between the dissimilarity of *Frankia* compositions and spatial distance (at site level: $r = -0.1037$, $P = 0.6967$; at area level: $r = 0.4897$, $P = 0.1833$). This suggested that spatial structure of *Frankia* compositions was unlikely to be resulted from spatial autocorrelation.

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

Discussion

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

Frankia diversity and composition on small spatial scales

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

× G: genotype–by–genotype interactions), (2) genetic trade-offs between bacterial strains, and (3) different functions of multiple *Frankia* genotypes.

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

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

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

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

Spatial structure in local genetic communities of *Frankia*

The results also revealed a complex, mosaic-like, genetic structures of *Frankia* on a single-host–population scale (spatial differentiation of *Frankia* OTU components; Fig 3) that did not depend on geographic distance. The explanations mentioned in the above section can also contribute to the understanding of the spatial mosaic-like patterns observed in *Frankia* genetic communities. The heterogeneous spatial structure of the interactions between genetically diverse hosts and rhizo-microorganisms can exert selective pressure resulting in the spatial differentiation of *Frankia* communities. In fact, we detected a genetic differentiation of the alder host not only among the studied riparian areas but also within each area (Kagiya et al. 2018), as well as the spatial genetic structure of *Frankia*. However, a part of patterns in genetic structure of *Frankia* were inconsistent with the pattern of host genetic structure. For example, *Frankia* compositions were different between BT and SE area (Fig. 5), while *A. hirsuta* populations were closely-related. BT area is completely covered with natural forest stands but SE area is close to agriculture field and its riverside landscape is artificially modified. The differences in abiotic and biotic environments could affects the selection outcomes (e.g., G × G × E: genotype–by–genotype–by–environment interactions), which may contribute the observed mosaic-like structures of the *Frankia* communities.

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

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

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

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Author contribution

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

Data availability statement

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

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Tables

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**

Figure legends

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

Fig 2. Phylogenetic tree based on the *nif*D-K spacer region in *Frankia*. A maximum-likelihood phylogeny was generated based on 97 sequences of the *nif*D-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* ('EAN1pec'), *Datisca glomerate*, 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.

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

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

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

colour figure

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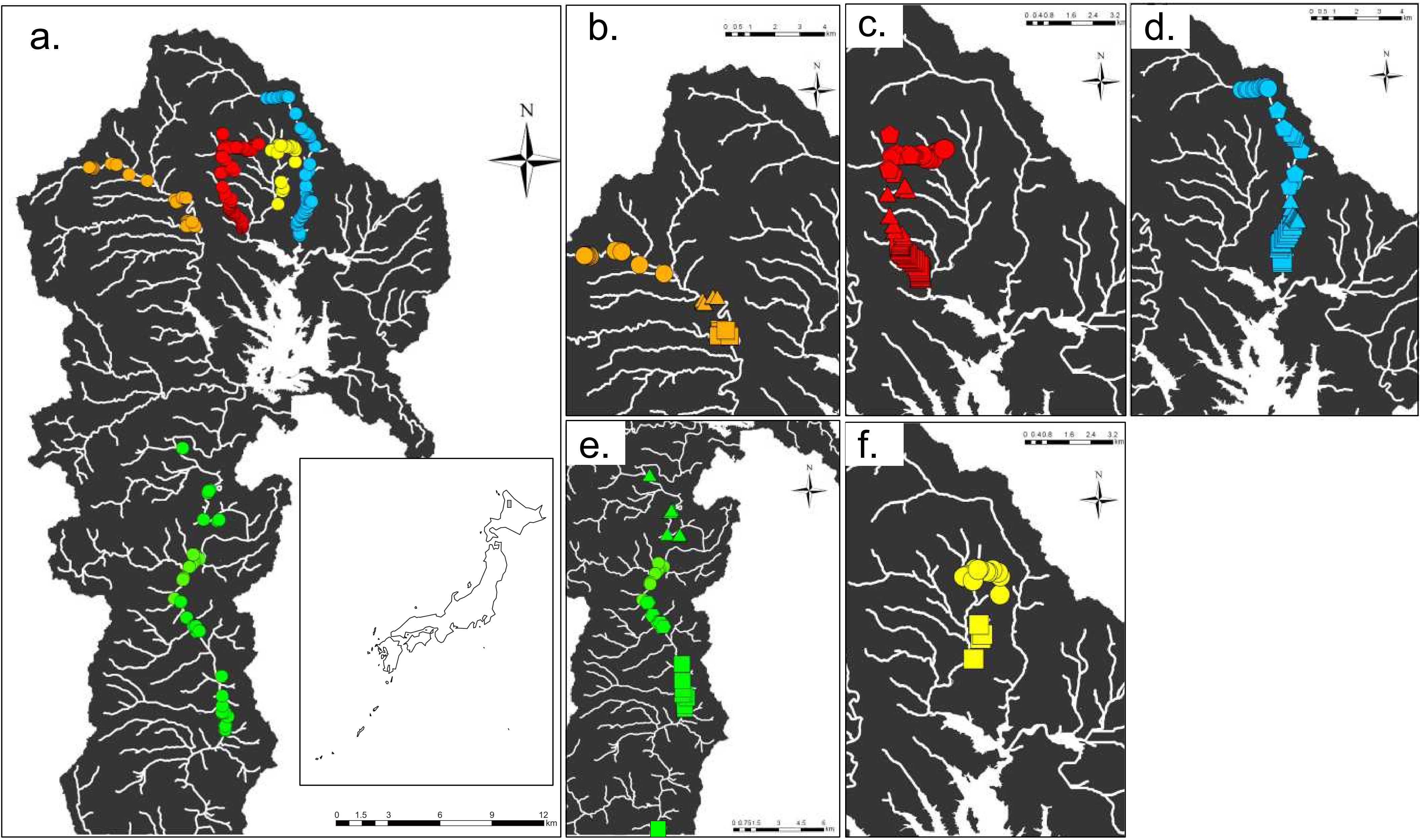

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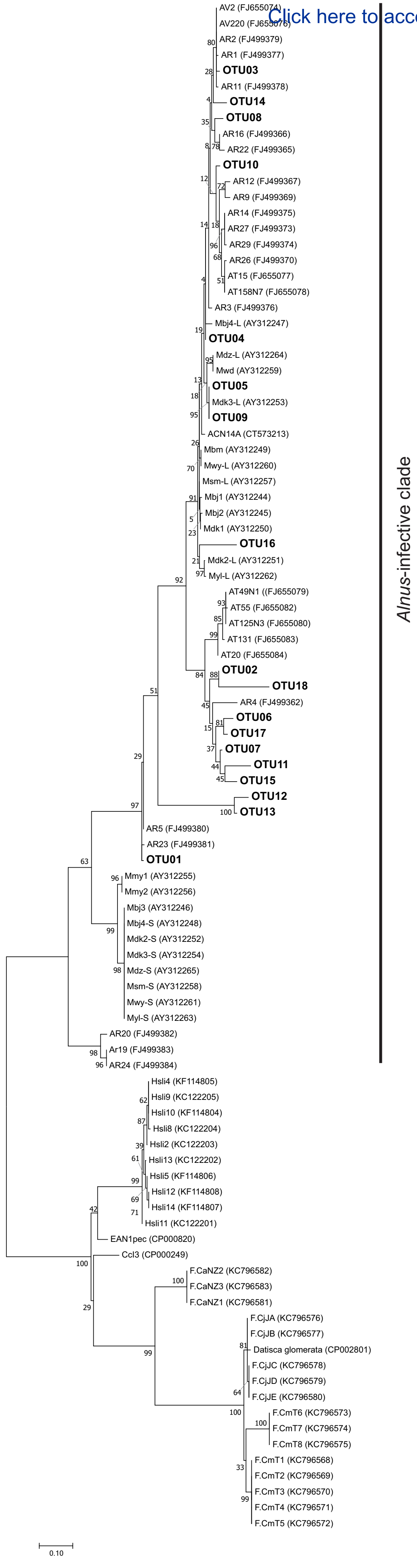

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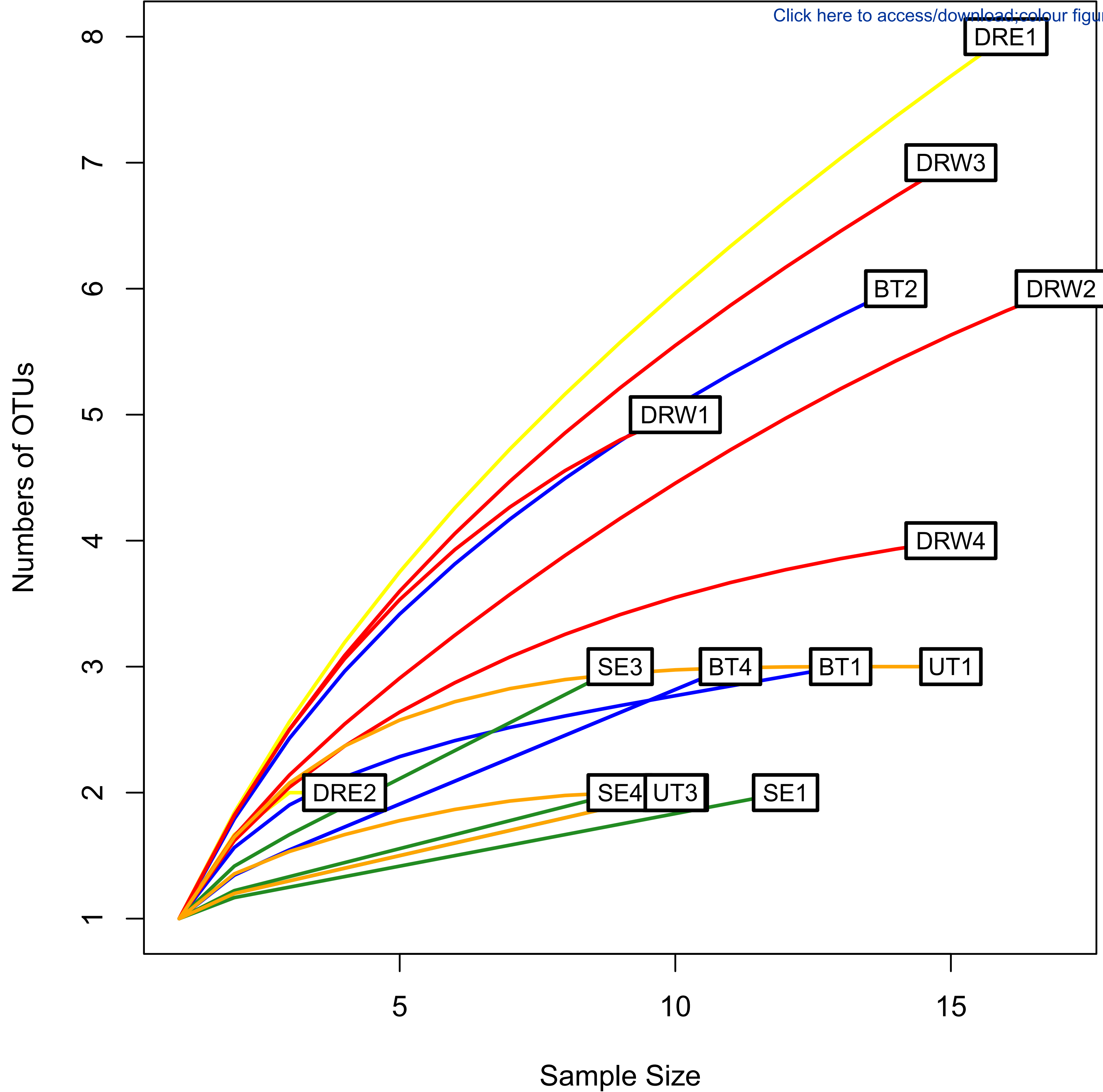

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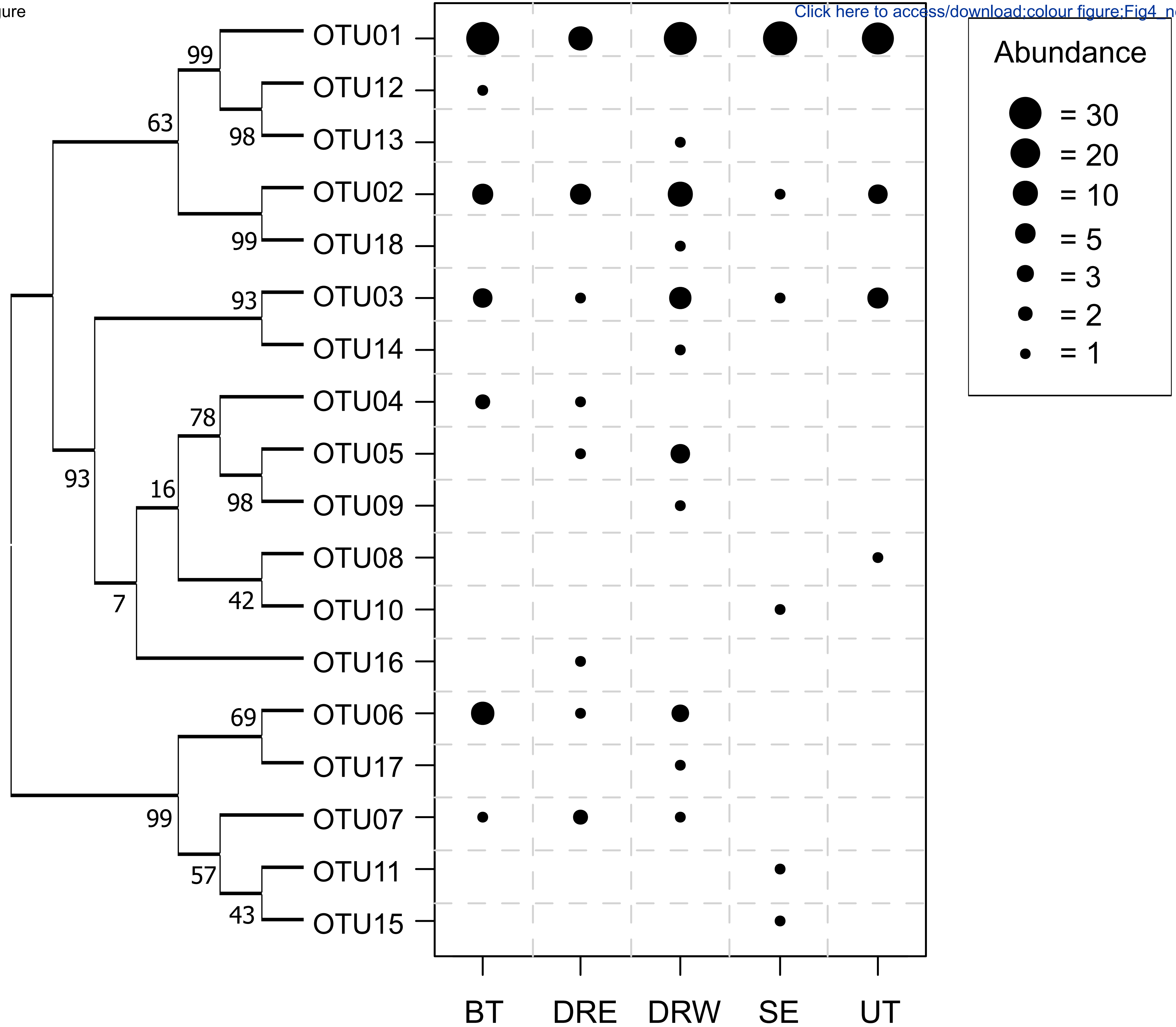

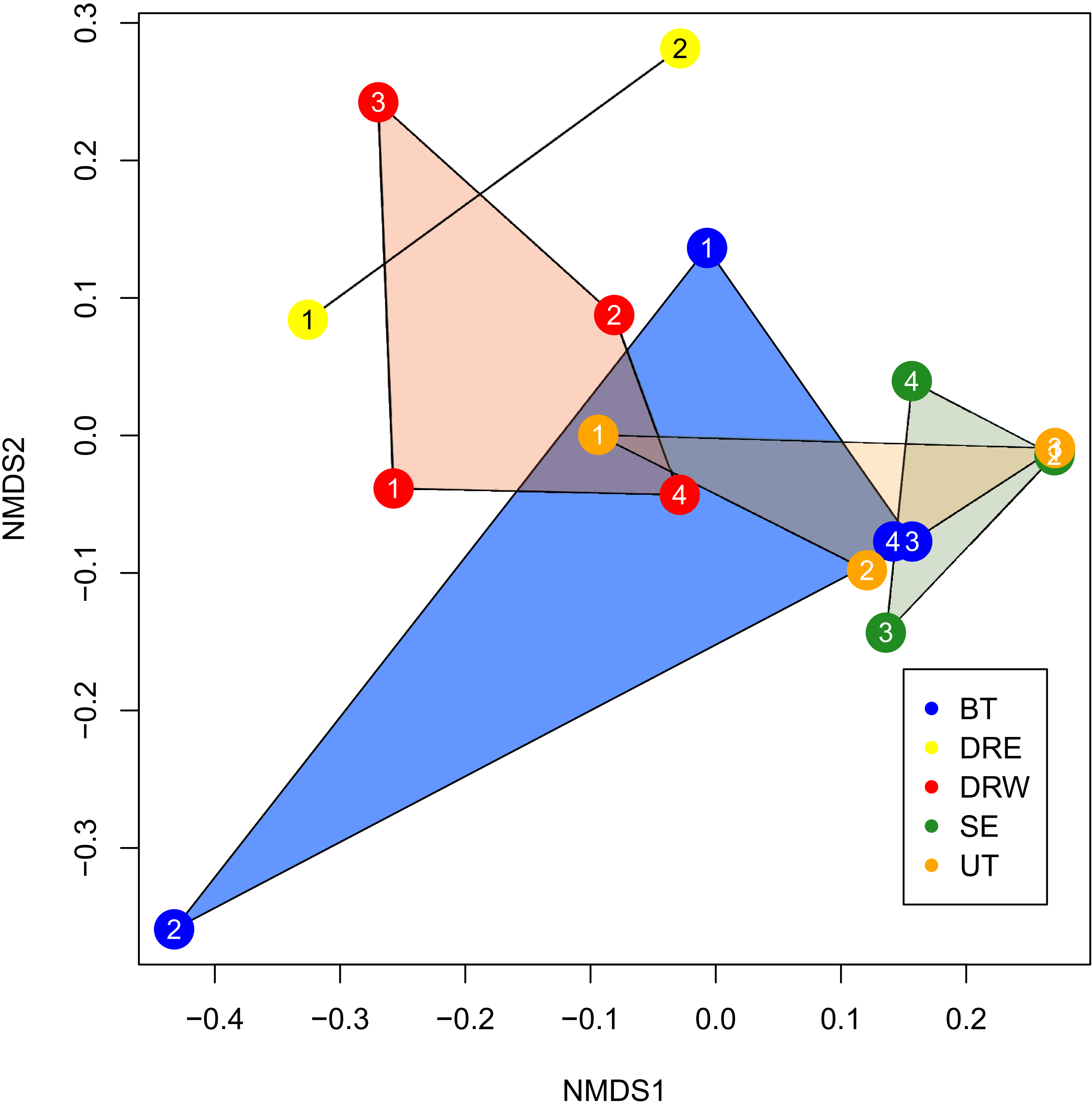
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Article title: High genetic diversity of bacterial symbionts in a single host species population: an alder–Frankia system in the field

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Table S1. Numbers of OTUs obtained from each site.

Area	BT				DRE		DRW				SE				UT			
Site No.	1	2	3	4	1	2	1	2	3	4	1	2	3	4	1	2	3	Accession No.
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	49
	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 *nif*D-K spacer region in *Frankia*. The neighbor-joining phylogeny was generated based on 97 sequences of the *nif*D-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* ('CcI3') 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.

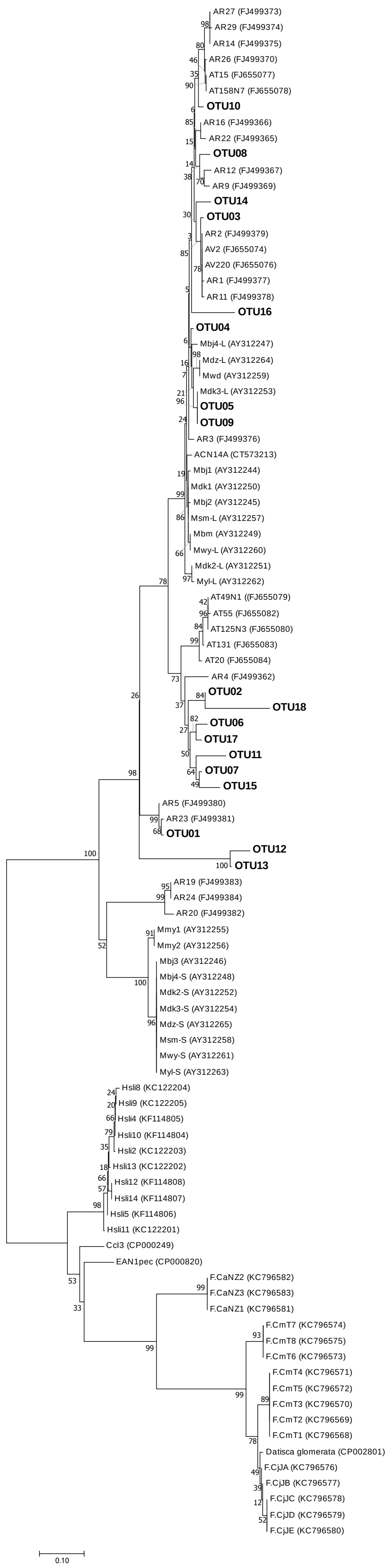
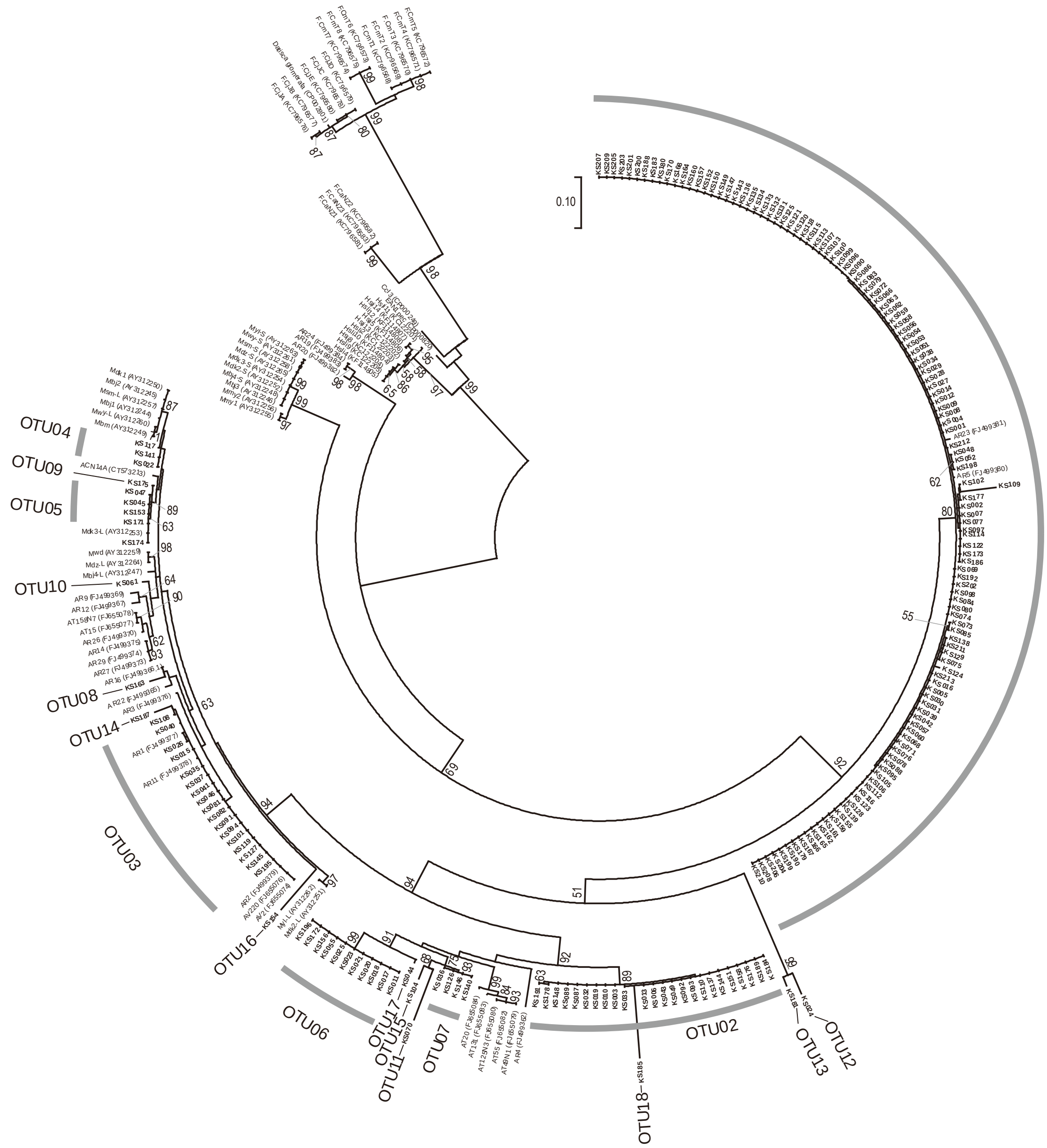

AR27 (FJ499373)
AR29 (FJ499374)
AR14 (FJ499375)
AR26 (FJ499370)
AT15 (FJ655077)
AT158N7 (FJ655078)
OTU10
AR16 (FJ499366)
AR22 (FJ499365)
OTU08
AR12 (FJ499367)
AR9 (FJ499369)
OTU14
OTU03
AR2 (FJ499379)
AV2 (FJ655074)
AV220 (FJ655076)
AR1 (FJ499377)
AR11 (FJ499378)
OTU16
OTU04
Mbj4-L (AY312247)
Mdz-L (AY312264)
Mwd (AY312259)
Mdk3-L (AY312253)
OTU05
OTU09
AR3 (FJ499376)
ACN14A (CT573213)
Mbj1 (AY312244)
Mdk1 (AY312250)
Mbj2 (AY312245)
Msm-L (AY312257)
Mbm (AY312249)
Mwy-L (AY312260)
Mdk2-L (AY312251)
Myl-L (AY312262)
AT49N1 ((FJ655079)
AT55 (FJ655082)
AT125N3 (FJ655080)
AT131 (FJ655083)
AT20 (FJ655084)
AR4 (FJ499362)
OTU02
OTU18
OTU06
OTU17
OTU11
OTU07
OTU15
AR5 (FJ499380)
AR23 (FJ499381)
OTU01
OTU12
OTU13
AR19 (FJ499383)
AR24 (FJ499384)
AR20 (FJ499382)
Mmy1 (AY312255)
Mmy2 (AY312256)
Mbj3 (AY312246)
Mbj4-S (AY312248)
Mdk2-S (AY312252)
Mdk3-S (AY312254)
Mdz-S (AY312265)
Msm-S (AY312258)
Mwy-S (AY312261)
Myl-S (AY312263)
Hsli8 (KC122204)
Hsli9 (KC122205)
Hsli4 (KF114805)
Hsli10 (KF114804)
Hsli2 (KC122203)
Hsli13 (KC122202)
Hsli12 (KF114808)
Hsli14 (KF114807)
Hsli5 (KF114806)
Hsli11 (KC122201)
CcI3 (CP000249)
EAN1pec (CP000820)
F.CaNZ2 (KC796582)
F.CaNZ3 (KC796583)
F.CaNZ1 (KC796581)
F.CmT7 (KC796574)
F.CmT8 (KC796575)
F.CmT6 (KC796573)
F.CmT4 (KC796571)
F.CmT5 (KC796572)
F.CmT3 (KC796570)
F.CmT2 (KC796569)
F.CmT1 (KC796568)
Datisca glomerata (CP002801)
F.CjJA (KC796576)
F.CjJB (KC796577)
F.CjJC (KC796578)
F.CjJD (KC796579)
F.CjJE (KC796580)
0.10

Fig S2. Phylogenetic tree based on all the *nif*D-K sequences of *Frankia*. A maximum-likelihood phylogeny was generated based on 280 sequences of the *nif*D-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 glomerate*, 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.


0.10
OTU01
OTU02
OTU03
OTU04
OTU05
OTU06
OTU07
OTU08
OTU09
OTU10
OTU11
OTU12
OTU13
OTU14
OTU15
OTU16
OTU17
OTU18